Adrenal tumors

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ADRENAL TUMORS

optimization of diagnostic strategies and patient management

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Adrenal tumors

optimization of diagnostic strategies and patient management

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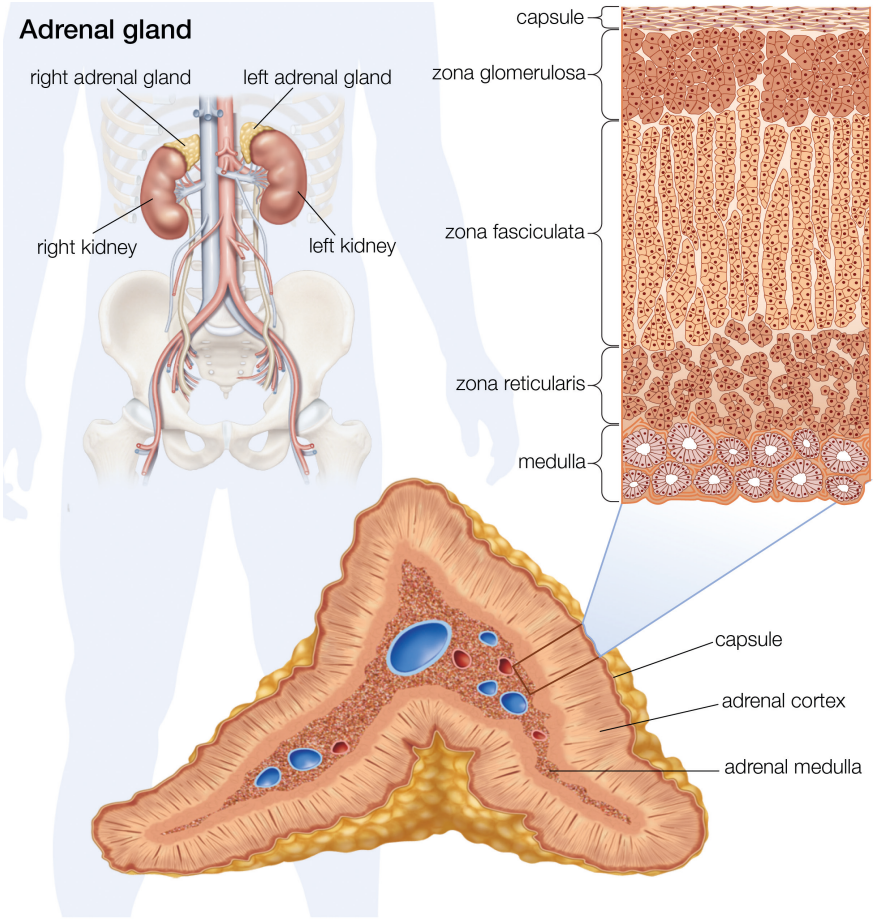
Contents

General introduction

The adrenal glands

The adrenal glands are endocrine organs situated in the retroperitoneum just above the kidneys (Figure 1). They consist of two histologically distinct parts, each with its own specific function. The outer layer, the adrenal cortex, is embryologically derived from the mesoderm and is able to synthesize and secrete steroid hormones. The inner part of the adrenal gland, i.e. the adrenal medulla, originates from the neural crest and consists of chromaffin cells which have the capacity to produce catecholamines, namely epinephrine, norepinephrine and dopamine.

Figure 1: Anatomy of the adrenal glands.



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Steroidogenesis

The adrenal cortex consists of three histological distinguishable zones. A distinct class of steroid hormones is synthesized in each zone, i.e. mineralocorticoids, glucocorticoids and sex steroids in the zona glomerulosa, zona fasciculata and zona reticularis, respectively. Biosynthesis of these hormones is a complex but extensively studied process in which cholesterol, the building brick of steroid hormones, is converted through a series of specific enzymatic steps into active hormones (Figure 2) (1,2). Secreted steroid hormones exert their function in various target tissues and are involved in many physiological processes such as the regulation of fluid and electrolyte balance, intermediate metabolism and, immune and stress responses (3).

Figure 2: Schematic representation of steroidogenesis.

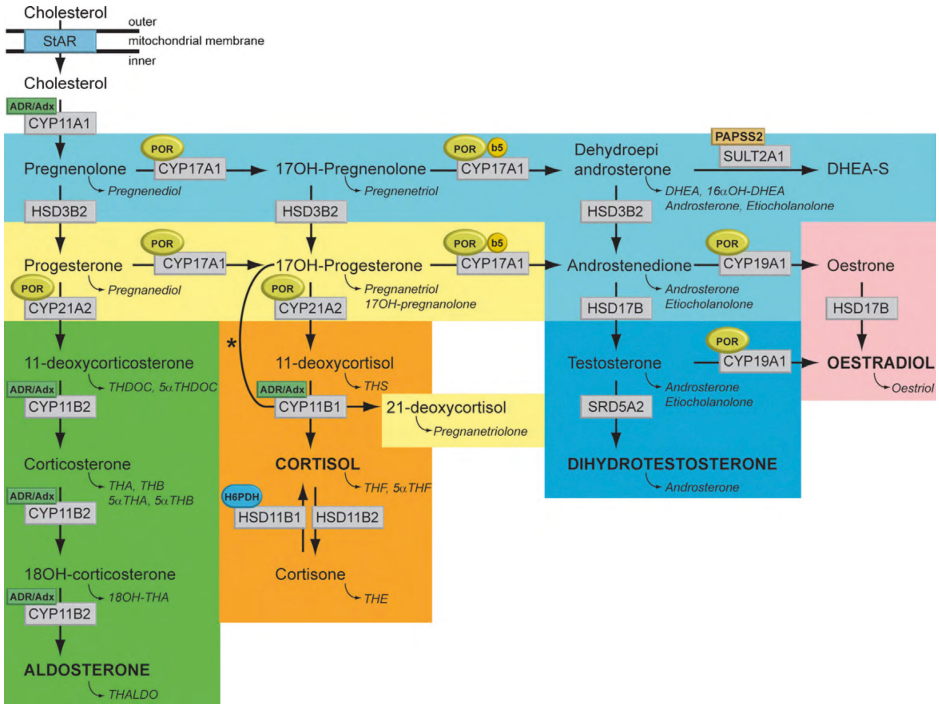


Figure from: Krone et al. J Steroid biochem Mol Biol 2010.

Steroidogenesis can be comprehensively evaluated using urinary steroid profiling (4,5). This chromatography-based technique, in which a wide variety of steroid hormone metabolites are measured in a 24 hour urinary sample, has been in use

and continuously improved since the 1960s. The clinical application of urinary steroid profiling is mainly to detect disorders that disturb steroidogenesis such as inborn steroid biosynthetic enzyme deficiencies, licorice- induced hypertension and hirsutism (5,7). Notably, recent studies suggest that urinary steroid profiling is able to discriminate between benign and malignant adrenal cortical tumors (8,9).

Whilst urinary steroid profiling measures the output of steroidogenesis, the input of steroidogenesis, which can be conceptualized as cellular influx of cholesterol into the steroidogenic cell and subsequent trafficking to the mitochondria for further processing, is understood mostly from inferential evidence (10). Circulating lipoproteins are believed to be a major source of cholesterol for steroidogenesis (11-13). This is mainly supported by the attenuated adrenal function in both humans and rodents with genetically determined abnormalities in lipoprotein synthesis or receptor mediated metabolism (14,15). The extent to which low density lipoproteins (LDL) and high density lipoproteins (HDL), lipoproteins that carry most of the cholesterol in the blood, contribute to steroidogenesis has been a matter of debate.

Diagnostic strategies in adrenal tumors

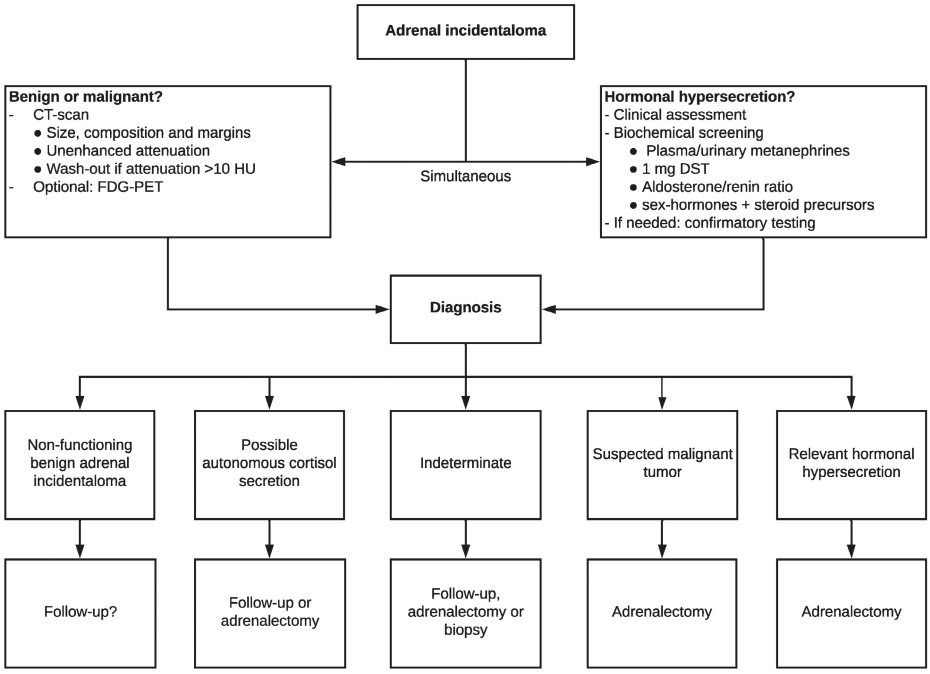
Tumors originating from the adrenal gland have a wide variety of etiologies including benign and malignant disorders originating from either the adrenal cortex or adrenal medulla (16,17). In addition, extra-adrenal malignant tumors can metastasize to the adrenal glands. Both adenomas and adrenocortical carcinomas can autonomously produce excessive amounts of one or more classes of steroid hormones. For example, overproduction of cortisol results in Cushing's syndrome and overproduction of aldosterone in primary aldosteronism. Tumors originating from the adrenal medulla are called pheochromocytomas and have the capacity to synthesize excessive amounts of catecholamines (18). Hormonal hypersecretion as well as adrenocortical carcinoma is associated with significant morbidity and mortality and these diagnoses should not be missed as most patients benefit from swift treatment, usually by surgical resection of the adrenal tumor (19-22). In case of a non-producing benign adrenal tumor follow-up is usually instituted.

A significant proportion of adrenal tumors is detected incidentally during imaging procedures performed for reasons not related to evaluation of the adrenal gland. These serendipitously discovered tumors are called adrenal incidentalomas. Radiological studies report a prevalence of 3-10% of adrenal incidentalomas with

the highest rates among the elderly (23,24). Guidelines on the management of adrenal incidentalomas recommend to evaluate whether hormonal hypersecretion is present by performing biochemical tests and if the adrenal incidentaloma is benign or malignant using imaging techniques (Figure 3) (16,17).

The biochemical analysis encompasses a 1 mg dexamethasone overnight suppression test to rule out autonomous cortisol production, assessment of the plasma aldosterone-renin ratio to evaluate primary aldosteronism (in hypertensive patients only), and measurement of plasma or urinary metanephrines (the *O*-methylated metabolites of catecholamines) to rule out pheochromocytoma.

Figure 3: Flowchart of the management of patients with an adrenal incidentaloma.



HU: Hounsfield Units, DST: Dexamethasone Suppression Test.

A diagnostic test with a high overall diagnostic accuracy to discriminate between a benign and malignant adrenal lesion is currently lacking. Therefore risk assessment is performed based on CT-phenotype. Unenhanced CT-scanning is primarily used to classify the tumor as either benign or of uncertain etiology (17). The attenuation value, measured on an unenhanced CT-scan, reflects the X-ray intensity relative to water and is able to differentiate between various tissues in the body (25). Adrenal

tumors with an unenhanced attenuation ≤10 Hounsfield Units (HU) are usually considered benign adenomas. Adrenal tumors with an attenuation value >10 HU cannot be classified as benign with certainty. A recent meta- analysis demonstrated that the 10 HU threshold has a sensitivity and specificity of 100% (95% CI: 91-100%) and 72% (95% CI: 60-82%), respectively (26). However, these numbers are based on only two studies with a total sample size of 41 cases.

In general, the adrenal incidentaloma population is characterized by a low prevalence of clinically relevant disease. Eventually, more than 70% of patients is diagnosed with a benign, non-functioning adenoma (17). Establishing this diagnoses requires repeated diagnostic testing with suboptimal diagnostic accuracy leading to a significant impact on healthcare resources, a high patient burden including potential anxiety. Remarkably there is still a lack of large high-quality prospective studies in this field and, therefore, the evidence based strength of most recommendations in the most recent guideline issued by the European Society of Endocrinology/European Network for the Study of Adrenal Tumors is either graded as low or very low (17). This implies that the optimal diagnostic strategy is still under debate. Improvement of diagnostic strategies should be considered very relevant, particularly in view of the vast number of potential patients due to the ever increasing application of imaging techniques (27).

Pheochromocytoma, sympathetic paraganglioma and hemodynamic instability

Pheochromocytomas, by definition originating from the adrenal medulla, have a histopathologically and functional counterpart that originates from the extra-adrenal sympathetic paraganglia. These sympathetic paragangliomas are mainly located paravertebral in the thorax, abdomen and pelvic region. Pheochromocytomas and sympathetic paragangliomas (PPGL) are considered to represent the same biological entity. PPGL have the capacity to produce excessive amounts of catecholamines (28).

In normal physiology, norepinephrine acts as a postsynaptic neurotransmitter of the sympathetic nervous system by activating α-adrenergic receptors located directly on the target tissue. Upon sympathetic stimulation both norepinephrine and epinephrine are also released from the adrenal medulla into the circulation where they exert their effect through stimulation of α- and β-adrenergic receptors. Importantly, the interaction between catecholamines and their receptors plays a

major role in the regulation of vascular tone and cardiac function (29).

Continuous or paroxysmal hypersecretion of catecholamines by PPGL is associated with severe cardiovascular morbidity and increased mortality (19,30). Surgical resection of the tumor offers the only curative treatment. However, surgery is considered a potentially hazardous procedure in these patient since many factors, such as anesthetic drugs, tracheal intubation or tumor manipulation, are known to evoke a sudden increase in catecholamine secretion (29). This can result in hemodynamic instability characterized by large variations in blood pressure and heart rate which seriously enhance the risk of cardiovascular complications. Treatment prior to PPGL resection with an α-adrenergic receptor blocker that inhibits the α-receptor mediated vasoconstrictive effects of catecholamines has been part of routine medical care for decades and it is believed pretreatment has contributed significantly to the improvement of patient outcome (31). Two frequently prescribed drugs for this purpose are phenoxybenzamine, a nonselective and noncompetitive α1- and α2- adrenergic receptor blocker, and doxazosin a selective and competitive α1-adrenergic receptor blocker. Previous studies comparing these drugs have yielded contradictory results regarding the duration and the magnitude of blood pressure deviation outside a certain target range as primary outcome measure (32-35). However, all studies thus far are seriously biased by a retrospective design, unstandardized pretreatment protocol and the lack of a standardized intraoperative management protocol to control hemodynamic fluctuations. The latter is an important factor to consider since anesthesiologists actively influence hemodynamic variables by the administration of vasoactive drugs and intravenous fluids. Moreover, it has been shown that deviation of blood pressure and heart rate from normal levels as well as the amount of vasoactive drugs that is administered intraoperatively to correct these deviations are associated with adverse postoperative outcome (31,36,37). Therefore, both hemodynamic deviations as well as corrective interventions should be considered as relevant markers of hemodynamic instability. A clinical tool to quantify the degree of hemodynamic instability by incorporating both factors is currently unavailable.

Aims and outline of the thesis

The aim of the present thesis is to improve diagnostic strategies that intend to differentiate clinically relevant adrenal tumors from those without clinical consequence and to optimize preoperative treatment for controlling intraoperative hemodynamic instability in patients with a pheochromocytoma or sympathetic paraganglioma.

Steroidogenesis is likely to be disrupted in adrenocortical carcinomas due to dedifferentiation. Retrospective studies suggested that urinary steroid profiling is able to detect disrupted steroidogenesis and might therefore be used to discriminate between benign and malignant adrenal lesions (8,9). Factors such age and sex are also known to influence steroidogenesis and might confound the diagnostic accuracy. In **Chapter 2** we described the validation of urinary steroid profiling using a novel gas chromatography with tandem mass spectrometry detection method (GC-MS/MS). Additionally, the influence of age, sex and oral contraceptive use on steroid profiling was determined and reference intervals were defined to enhance diagnostic accuracy. Previous studies suggested that steroidogenesis is altered in subjects with genetically determined low plasma HDL-C but the results of different studies are contradictory (13,38). However, little is known about the relationship between circulating lipoproteins and steroidogenesis in the general population. These observations prompted us to explore the relationship between total glucocorticoid production using a GC-MS/MS method and plasma HDL-C in a cross-section study involving 240 healthy subjects from the general population as described in **Chapter 3**. We hypothesized that total glucocorticoid production would be decreased in subjects with low HDL-C levels. In **Chapter 4** we presented the measurements of circulating lipoprotein concentrations in blood samples taken from the adrenal vein and inferior vena cava during adrenal venous sampling procedures in patients with primary aldosteronism. We hypothesized that uptake of circulating lipoproteins in the adrenal gland could be estimated using this model.

Reliable information on the incidence of various etiologies is needed for optimization of diagnostic strategies in adrenal tumors. The sensitivity of biochemical and imaging techniques to detect PPGL has improved substantially and this is likely to affect the detection rate of these tumors (39-41). In **Chapter 5** we investigated the annual incidence of pheochromocytoma and sympathetic paraganglioma in the Netherlands between 1995 and 2015. Additionally we assessed trends in incidence by comparing our results with historical numbers derived from a systematic review of the literature. Further improvement of diagnostic strategies to distinguish between clinically relevant adrenal tumors from those without clinical consequence can be achieved by incorporating new diagnostic modalities, such as urinary steroid profiling, or by extending the application of already performed diagnostic tests. For example, small-sized studies have suggested that pheochromocytomas are characterized by an attenuation value >10 HU on unenhanced CT-scanning and that biochemical testing might

thus be obviated in patients with an attenuation value ≤10 HU. Therefore, in **Chapter 6** we examined the diagnostic accuracy of unenhanced CT scanning to exclude a pheochromocytoma in case of adrenal tumor. Data were collected retrospectively in several Dutch centers and included central revision of all CT-scans. In **Chapter 7** we conducted a meta-analysis on the same topic to confirm the diagnostic accuracy of unenhanced CT-scanning in an unprecedented large number of pheochromocytomas. Additionally, we performed a cost evaluation of a newly proposed diagnostic strategy in which biochemical testing to rule out a pheochromocytoma would only be performed in patients harboring an adrenal incidentaloma with an unenhanced attenuation value >10 HU.

Preoperative treatment of patients with PPGL is routinely instituted to prevent cardiovascular complications. To accomplish this aim clinicians strive to maintain a stable perioperative hemodynamic course. Evaluation of hemodynamic instability as a reliable outcome measure requires complex integration of many factors that are considered to represent a part of the hemodynamic instability spectrum. Currently, a tool for comprehensive assessment of hemodynamic instability is lacking. In **Chapter 8** we described the development and validation of a scoring method that rates the degree of hemodynamic instability. This so called hemodynamic instability score (HI-score) incorporates hemodynamic variables as well as applied interventions to correct hemodynamic variables. We conducted the very first randomized controlled trial that compares the efficacy of preoperative treatment with two commonly used α-adrenergic receptor blockers in patients with PPGL. The result are described in **Chapter 9.**

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PART I

Adrenal cortex: evaluation of adrenal steroidogenesis and its relationship with lipoproteins

Chapter 2

Determination of reference intervals for urinary steroid profiling using a newly validated GC-MS/MS method

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Abstract

Background: Urinary steroid profiling (USP) is a powerful diagnostic tool to asses disorders of steroidogenesis. Pre- analytical factors such as age, sex and use of oral contraceptive pills (OCP) may affect steroid hormone synthesis and metabolism. In general, USP reference intervals are not adjusted for these variables. In this study we aimed to establish suchreference intervalsusing anewly-developed and validated gas chromatography with tandem mass spectrometry detection method (GC-MS/MS).

Methods: Two hundred and forty healthy subjects aged 20–79 years, stratified into six consecutive decade groups each containing 20 males and 20 females, were included. None of the subjects used medications. In addition, 40 women aged 20–39 years using OCP were selected. A GC-MS/MS assay, using hydrolysis, solid phase extraction and double derivatization, was extensively validated and applied for determining USP reference intervals.

Results: Androgen metabolite excretion declined with age in both men and women. Cortisol metabolite excretion remained constant during life in both sexes but increased in women 70–79 years of age. Progesterone metabolite excretion peaked in 30–39-year-old women and declined afterwards. Women using OCP had lower excretions of androgen metabolites, progesterone metabolites and cortisol metabolites. Method validation results met prerequisites and revealed the robustness of the GC-MS/ MS method.

Conclusions: We developed a new GC-MS/MS method for USP which is applicable for high throughput analysis. Widely applicable age and sex specific reference intervals for 33 metabolites and their diagnostic ratios have been defined. In addition to age and gender, USP reference intervals should be adjusted for OCP use.

Introduction

Steroid biosynthesis is a complex process by which steroid hormones are produced from cholesterol through a series of unique enzymatic steps in steroidogenic tissue (1). This tissue is mainly found in the adrenal cortex and gonads (2). Steroid hormones can be classified according to their physiological function into progestins, androgens, estrogens, mineralocorticoids and glucocorticoids. As such, they regulate various biological processes including mineral balance, intermediate metabolism, sexual development, reproductive function, immune and stress responses (3). Steroids are converted into a large number of metabolites by the liver and peripheral tissues before being excreted in the urine. The biochemistry of steroid biosynthesis and metabolism is largely known and specific steroid pathways are regulated by differential expression and activity of enzymes and cofactors involved in a developmental, sex, time and tissue specific fashion and might be perturbed in disease states (4).

Since the 1960s, urinary steroid profiling (USP) has been a powerful diagnostic tool to assess steroidogenesis. Nowadays, USP is usually being performed by application of gas chromatography-mass spectrometry (GC-MS) (4,5). This technique is able to measure a wide variety of urinary steroid hormone metabolites at the same time in one urinary sample, making it an efficient and patient friendly diagnostic tool. For the last 50 years almost all disorders of steroid hormone biosynthesis and metabolism have been characterized and first named following urinary steroid analysis (4).

USP has a broad range of applications. For example, it can be used for the diagnosis and follow-up of disorders resulting from steroid biosynthetic enzyme deficiencies, licorice-induced hypertension, hirsutism and other related diseases (6–8). Furthermore, USP might be helpful in monitoring patients with an adrenocortical carcinoma (ACC) and could be useful in discriminating between malignant and benign adrenal tumors (9–12). Reference intervals for USP using GC-MS have been described before (6,11,13–17). Notably, those previous studies have several shortcomings, such as lack of adjustment for potential relevant pre-analytical factors like age, sex or use of oral contraceptive pills (OCP) or limited external validity as a result of the examination of study subjects who may not accurately reflect the general population. Reliable reference intervals for urinary steroid metabolites are a prerequisite for correct interpretation of USP test results in clinical practice.

In this study we aim to establish age- and sex-specific USP reference intervals in a well-defined healthy adult population. In addition, USP reference intervals were determined in a subgroup of women using OCP. USP was performed by using a newly developed gas chromatography-tandem mass spectrometry (GC-MS/MS) assay. In comparison with other USP methods such as GC-MS, it has been suggested that GC-MS/MS demonstrates higher specificity, while being less laborious and more suitable for high-throughput analysis.

Materials and methods

Subjects

Two hundred and eighty healthy subjects with a body mass index between 21 and 30 kg/m^2 and age between 20 and 79 years were selected from the Life Lines Cohort Study, a large population- based cohort study in the Netherlands (18). Of these, 240 subjects were stratified into six consecutive decade groups, each containing 20 males and 20 females. None of the subjects used any medication. In the subgroup of women between 20 and 39 years who were not using OCPs, any women using OCP were excluded.

In addition, 40 women aged 20–39 years (20 subjects per decade), using OCP were selected. Women on OCP used combined contraceptives with different progestogens, but mostly levonogestrel combined with ethinylestradiol. Urinary samples from 24 h collections had been stored at −80 °C until analysis. In women, urinary collections were not timed according to menstrual cycle or day of OCP use. The study was approved by the Medical Ethics Committee of the University of Groningen and all participants provided written informed consent.

Reagents and stock solutions

Methoxyamine HCl, trimethylsilylimidazole (TMSI) and sodium ascorbate were purchased from Sigma Aldrich Corp. (St. Louis, MO, USA). Pyridine was obtained from Merck (Kenilworth, NJ, USA), heptane and methanol from Biosolve BV (Valkenswaard, The Netherlands), and Suc d'Helix from Brunschwig Chemie (Amsterdam, The Netherlands).

Androsterone (A), etiocholanolone (E), dehydroepiandrosterone (DHEA), 11-keto-etiocholanolone (11-KE), 11-hydroxyandrosterone (11- HA), 11-hydroxyetiocholanolone (11-HE), epipregnanolone (polone), 16-α hydroxydehydroepiandrosterone (16-OH-DHEA), allo-pregnanediol (aP2), pregnanediol (P2), pregnanetriol (P3),

16-ketoandrostenediol (16-KA'2), androstenetriol (A'3), tetrahydrodeoxycortisol (THS), 11-deoxytetrahydrocorticosterone (TH-DOC), pregnanetriolone (PTL), 16-α hydroxypregnenolone (16-OH-P'OL), 17-hydroxypregnenetriol (17-P3), tetrahydrocortison (THE), 11-dehydrotetrahydrocorticosterone (THA), tetrahydrocorticosterone (THB), allo-tetrahydrocorticosterone (aTHB), tetrahydrocortisol (THF), allo-tetrahydrocortisol (aTHF), α cortolone (α-CTLN), β cortolone (β-CTLN), pregnanediolone (PDL) and allo-pregnanediolone (aPDL) were all obtained from Steraloids (Newport, RI, USA). Estriol and α-cortol (α-cortol) were from Sigma Aldrich Corp. (St. Louis, MO, USA). See Supplementary Table 1 for steroid nomenclature according to IUPAC, LOINC and Chemspider.

Isotope-labeled internal standards 11-KE-d5, pregnenolone- d4 and THE-d5 were purchased from CDN isotopes; DHEA-d6 from Sigma Aldrich Corp. (St. Louis, MO, USA). We used four deuterated internal standards divided over the 33 steroid metabolites representing polarity groups, because of availability and costs. For 3α, 15β, 17α-trihydroxypregnanediolone (15-OH-PDL), 15-hydroxypregnenolone (15-OH-P'DL) and 16-β,18-dihydroxydehydroepiandrosterone (16,18-OH_2-DHEA) we have no standards available. Stock solutions were prepared in methanol and serially diluted to form calibrators and quality control samples in urine by enrichment. The exact concentration range of calibrators varies with the analyte, for example, 0–27 μmol/L for THF and 0–36 μmol/L for E. Internal standards concentrations were 6 μmol/L.

Instrumentation

Solid phase extraction (SPE) was performed on Waters Oasis HLB (3 mL Vac cartridges, 60 mg sorbent per cartridge, 30 μm particle size (Waters Corporation, Milford, MA, USA). Steroids were chromatographically separated on a J&W CP-Sil 5 CB column (25 m × 250 μm × 0.12 μm; Agilent Technologies, Santa Clara, CA, USA). A 7890A GC with 7000 Triple Quadrupole Detector (Agilent Technologies, Santa Clara, CA, USA) was used for separation and detection using electron impact and selective reaction monitoring. Nitrogen was used as collision gas (flow 1.5 mL/min), helium as quench gas (flow 2.25 mL/min) and carrier gas (2 mL/min). The injection temperature was 65 °C, with the MS source at 270 °C and both quadrupoles at 150 °C. Chromatography was performed using a temperature program for optimal separation: 2 min 50 °C, ramp 40 °C/min until 160 °C, ramp 2.5 °C/min until 240 °C and finally ramp 4 °C/min until 270 °C. Electron impact was performed at 70 eV.

Data acquisition was performed with Masshunter Version B 06.01 (Agilent

Technologies, Santa Clara, CA, USA) and data were processed with Masshunter Quantitative Analysis Version B07.00/ Build 7.0.457.0 (for QQQ).

Analytical principle

Glucuronide- and sulfate-conjugated steroid hormone metabolites were measured in samples from 24 h urine collections. First, conjugated hormones were converted to the free steroid form by enzymatic hydrolysis. Isotope-labeled internal standards 11-KE-d5, DHEA-d6, pregnenolone-d4 and THE-d5 were added and unconjugated steroids were extracted from urine by using SPE. Polar components were washed out of the extract. The extract was vaporized using an infrared vaporizer, after which the residue was derivatized at hydroxyl- and keto-residues, in a two-step reaction to decrease polarity. Keto-residues were derivatized using 2% methoxyamine in pyridine; hydroxyl-residues were silylated by N-trimethylsilyl imidazole.

Sample preparation

Before analysis, urinary samples were centrifuged at 1200 *g* before applying 1 mL to conditioned (methanol, water) HLB SPE columns. Cartridges were washed with water and eluted with methanol. The eluate was vaporized using an infrared vaporizer (Hettlab IR Dancer 300, Hettich AG, Switzerland) and rediluted in 2 mL acetate/sodium ascorbate (pH 4, 8) solution. One hundred microliters Suc d'Helix Pomatia was added and enzymatic hydrolysis of the conjugated groups took place during 2 h at 46 °C in a shaking temperature controlled bath. After cooling down, internal standards were added and a second SPE step took place on the HLB columns. Samples were washed with water, eluted with methanol and evaporated until dryness. The residue was derivatized with 150 μL methoxyamine in pyridine during 1 h at 80 °C. After evaporation until dryness a second derivatization step took place with 200 μL N-trimethylsilyl imidazole during 12 h (overnight) at 110 °C. In case of emergency diagnostics this last step can be reduced to 2 h at 140 °C. Samples were washed with 4 mL heptane and 3 mL 0.1 M HCl by vortexing and centrifugation (1200 *g*). One milliliters of the upper heptane layer were transferred to a GC-MS/MS vial. Injection volume was 25 μL.

Analytical method validation

Prior to validation, claims were postulated for each validation parameter, according to the international ISO15189 regulation and the Dutch guideline for validation of analytical methods in medical laboratories by the Dutch Society of Clinical Chemistry and Laboratory Medicine (NVKC) (19). Validation parameters applied were intra-

assay (n = 20) and inter-assay (n = 16) imprecision, repeatability of the injection (n = 10), linearity (n = 6), recovery (n = 6 for three concentrations), lower limit of quantitation (LLOQ), minimal sample volume, carry-over, method comparison against the previous GC-MS method using liquid-liquid extraction and overnight hydrolysis at 37 °C using a buffer without sodium ascorbate (7) and stability of six different urinary samples (biological sample, freeze- thaw, autosampler).

Statistical analysis

GC-MS/MS and GC-MS methods were compared using Passing- Bablok regression analysis in Analyse-it (version 2.30 Excel 12^{+} Analyse-it Software). USP results from healthy volunteers were analyzed to obtain age, sex and OCP specific reference intervals for 33 steroid metabolites. Also diagnostic steroid ratio reference intervals were calculated from these data. Reference intervals were defined as the central 95% of the population (i.e. the 2.5th and 97.5th percentiles) and calculated using EP evaluator. Non-parametric data were logarithmically transformed before analysis.

Differences in the distributions of urinary steroid metabolites in relation to OCP use were assessed using the Kolmogorov- Smirnov test. Metabolites were categorized in androgen (A, E, DHEA, 11-KE, 11-HA, 11-HE), cortisol (THE, THF, aTHF, α-CTLN, β-CTLN, α-cortol), progesterone (aP2, P2, P3, Polone), aldosterone (THA, THB, aTHB), intermediate (THS, PDL, PTL, aPDL, TH-DOC) and fetal (A'3, 16K-A'2, 16-OH-DHEA, 16,18-(OH)2-DHEA, 16-OH-p'ol, 15-OH-PDL, 17-P3, 15-OH-P'DL) metabolites. Sum scores were calculated per group and differences between groups were calculated using the Mann-Whitney test. Additional statistical analyses were performed using SPSS version 23.0 for Windows (IBM Corporation, Chicago, IL, USA). A two-sided $p < 0.05$ was considered to indicate statistical significance.

Table 1: Mass spectrometric settings for steroid metabolites and internal standards.

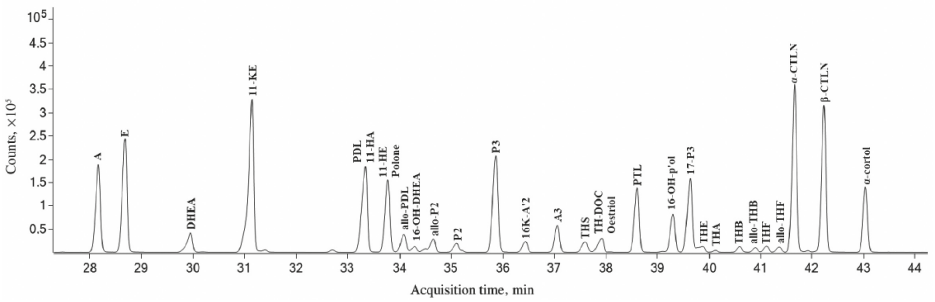
Steroid metabolite	Precursor ion, *m/z*	Product ion, *m/z*	Collision energy, V	Dwell time, ms	Internal standard
[D5] 11-KE	305.1	258.2	10	50	
[D6] DHEA	364.2	274	3	50	
[D5] THE	583.3	403.2	12	50	
[D4] Pregnenolone	390.2	300.1	7	30	
11-HA	448.2	358.2	3	20	D6-DHEA
11-HE	448.2	268.2	10	40	D6-DHEA
11-KE	300.1	254.2	10	50	D5-11-KE
15-OH-PDL	258	168.1	8	40	D5-THE
15-OH-P'DL	562	472.3	8	30	D5-THE
16,18-OH_2-DHEA	534	444	10	30	D5-11-KE
16K-A'2	446.2	356.2	7	40	D6-DHEA
16-OH-DHEA	266	239.1	10	40	D5-11-KE
16-OH-p'ol	474.3	156	18	10	D5-11-KE
17-P3	433.2	253.3	7	10	D5-THE
A	360.2	270.2	5	50	D4-Pregnenolone
A'3	329	239.1	15	30	D5-THE
aP2	269.2	187	3	30	D5-THE
aPDL	476.3	386.3	10	40	D5-THE
aTHB	474	384.3	5	100	D5-THE
aTHF	472	382.1	15	80	D5-THE
α-cortol	343.3	199	5	30	D5-THE
α-CTLN	449.2	359.3	5	30	D5-THE
β-CTLN	449.2	359.3	3	30	D5-THE
DHEA	358.2	268	3	50	D6-DHEA
E	360.2	270.2	5	50	D4-Pregnenolone

Table 1: Continued

Steroid metabolite	Precursor ion, *m/z*	Product ion, *m/z*	Collision energy, V	Dwell time, ms	Internal standard
Oestriol	504.3	414.1	3	40	D5-THE
P2	269.2	187	3	30	D5-THE
P3	435.3	255.2	5	20	D5-THE
PDL	476.3	386.3	10	40	D5-THE
Polone	388.2	298.2	10	40	D4-Pregnenolone
PTL	449.2	359.3	3	30	D5-THE
THA	400	241	5	30	D5-THE
THB	474	384.3	5	100	D5-THE
TH-DOC	476.3	241.2	10	30	D5-THE
THE	578.3	398.2	12	50	D5-THE
THF	472	382.1	15	80	D5-THE
THS	564.3	474.2	12	40	D5-THE

Abbreviations as in Figure 1 and Supplemental Table 1.

Figure 1: Total ion current chromatogram of a calibration standard mix of all the steroid metabolites.



A, androsterone; E, etiocholanolone; DHEA, dehydroepiandrosterone; 11-KE, 11-keto-etiocholanolone; 11-HA, 11-hydroxyandrosterone; 11-HE, 11-hydroxyetiocholanolone; polone, epipregnanolone; 16-OH-DHEA, 16-α hydroxydehydroepiandrosterone; aP2, allo-pregnanediol; P2, pregnanediol; P3, pregnanetriol; 16-KA'2, 16-ketoandrostenediol; A'3, androstenetriol; THS, tetrahydrodeoxycortisol; TH-DOC, 11-deoxytetrahydrocorticosterone; PTL, pregnanetriolone; 16-OH-P'OL, 16-α hydroxypregnenolone; 17-P3, 17-hydroxypregnenetriol; THE, tetrahydrocortison; THA, 11-dehydrotetrahydrocorticosterone; THB, tetrahydrocorticosterone; aTHB, allo-tetrahydrocorticosterone; THF, tetrahydrocortisol; aTHF, allo-tetrahydrocortisol; α-CTLN, α cortolone; β-CTLN, β cortolone; PDL, pregnanediolone; aPDL, allo-pregnanediolone; α-cortol, β-cortol. Standards for 3α, 15β, 17α-trihydroxypregnanediolone (15-OH-PDL), 15-hydroxypregnenolone (15-OH-P'DL) and 16-β,18-dihydroxy-dehydroepiandrosterone (16,18-OH_2-DHEA) were not available. See Supplementary Table 1 for steroid nomenclature according to IUPAC, LOINC and Chemspider.

Results

Validation parameters

In one chromatographic run, we quantified 33 urinary steroid metabolites, as shown in the total ion current chromatogram (Figure 1) and mass spectrometric settings (Table 1). Calibration curves (weighed regression) and validation samples were run with every batch of patient samples. Linearity was obtained over the 0–35 μmol/L range with corresponding correlation coefficients (R^2) consistently >0.99 for all steroids. Calibration curves were also reproducible between days (n = 6) with $R^2 > 0.95$. Coefficient of variation (CVs) of slopes between days were <3% (calibration data not shown).

Intra-assay imprecision (n = 20), inter-assay imprecision (n = 16) and repeatability imprecision (n = 10) were <10% except for 16-KA'2, which showed an intra- and inter-assay imprecision of 14% and 16%, respectively (data not shown). Recoveries (n = 6) measured by spiking urine samples with three different concentrations of

standard solution, ranged from 89% to 112%, as shown in Supplementary Table 2. The LLOQ, or functional sensitivity, was at least 0.1 μmol/L for each analyte with a CV < 20% (data not shown). The minimal sample volume was established to be 500 μL. The method did not suffer from carry-over (<0.1% for all analytes, data not shown). Primary urine samples were stable at room temperature for at least 1 week, at 4 °C for at least 8 weeks and at –20 °C for at least 12 weeks. Samples were stable for at least 4 freeze-thaw cycles. Derivatized samples were stable for at least 2 weeks in the autosampler (room temperature). Stability data are shown in Table 2.

Method comparison

We compared the results obtained by the newly-developed GC-MS/MS and the former GC-MS method in a series of patients specimens routinely analyzed for USP at our laboratory. Passing-Bablok regression (n = 20) showed slightly lower concentrations for A, 11-KE, PDL, 11-HA, 11-HE, aPDL, aP2, P2, P3, A'3, PTL, 17-P'3, THA, THB, aTHB, THF, aTHF and a-cortol when quantified with the new GC-MS/MS method compared to the GC-MS method, as shown in Supplementary Table 3.

Table 2. Stability of steroid metabolites (n = 6).

Steroid metabolite	Room temperature, days	4 °C, days	-20 °C, days	Freeze/thaw, cycles	Autosampler, days
A	>90	>90	>90	4	>14
E	83	>90	>90	4	>14
DHEA	25	>90	>90	4	>14
11-KE	24	>90	>90	4	>14
PDL	41	>90	>90	4	>14
11-HA	>90	>90	>90	4	>14
11-HE	>90	>90	>90	4	>14
Polone	Not tested	Not tested	Not tested	Not tested	>14
aPDL	Not tested	Not tested	Not tested	Not tested	>14
16-OH-DHEA	6	55	>90	4	>14
aP2	>90	>90	>90	4	>14
P2	>90	>90	>90	4	>14
P3	46	>90	>90	4	>14
16-KA'2	Not tested	Not tested	Not tested	Not tested	>14
A3	11	>90	>90	4	>14
THS	19	>90	>90	4	>14
Oestriol	Not tested	Not tested	Not tested	Not tested	>14
TH-DOC	Not tested	Not tested	Not tested	Not tested	>14
PTL	>90	>90	>90	4	>14
16-OH-p'ol	Not tested	Not tested	Not tested	Not tested	>14
17-P3	12	>90	>90	4	>14
THE	23	>90	>90	4	>14
THA	33	>90	>90	4	>14
THB	>90	>90	>90	4	>14
aTHB	>90	>90	>90	4	>14
THF	>90	>90	>90	4	>14
aTHF	>90	>90	>90	4	>14
α-CTLN	29	65	>90	4	>14
β-CTLN	37	55	>90	4	>14
α-cortol	>90	>90	>90	4	>14

Abbreviations as in Figure 1 and Supplemental Table 1.

Table 3: Reference intervals of urinary steroid metabolites in men (μmol/24 h) per decade.

Steroid metabolite	Age decade, years											
	20–29		30–39		40–49		50–59		60–69		70–79	
	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]
Androgen												
A	6.8–36.4	21.6	6.4–29.0	13.7(NP)	5.5–22.7	14.0	3.2–17.6	10.4	3.5–14.7	6.8(NP)	2.6–11.0	6.8
E	5.0–39.9	13.5(NP)	0.3–16.9	8.6	3.1–16.1	9.6	1.2–16.1	8.6	1.5–13.0	7.3	2.1–12.1	7.0
DHEA	0.5–41.2	6.6(NP)	0.2–17.3	1.5(NP)	0.6–12.9	2.6(NP)	0.2–7.0	1.1(NP)	0.2–3.2	0.6(NP)	0.1–7.8	0.9(NP)
11-KE	0.1–3.6	1.8	0.1–3.5	1.6	0.1–3.4	1.7	0.4–5.2	1.8(NP)	0.5–4.8	1.9(NP)	0.4–4.6	1.7(NP)
11-HA	1.1–9.5	5.3	1.1–6.9	4.0	2.3–7.2	4.8	2.2–8.1	4.2(NP)	0.9–8.2	4.5	1.6–7.3	4.4
11-HE	0.1–4.0	1.9	0.0–4.7	1.6(NP)	0.1–4.1	1.9	0.1–4.6	2.0	0.2–6.3	1.4(NP)	0.5–7.6	1.9(NP)
Estriol	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Cortisol												
THE	5.0–25.3	15.2	6.5–24.7	12.9(NP)	7.1–20.9	14.0	5.1–24.2	14.7	5.4–23.0	14.2	5.6–22.6	14.1
THF	3.5–13.5	6.9(NP)	3.1–10.0	6.5	3.8–10.6	7.2	2.5–13.4	7.9	3.9–16.0	8.2(NP)	4.0–15.3	9.7
aTHF	2.5–21.6	7.4(NP)	1.8–15.3	8.5	3.9–12.8	8.3	3.3–14.7	7.1(NP)	3.0–13.2	5.9(NP)	1.6–12.4	7.0
α-CTLN	2.2–8.5	5.4	2.7–6.7	4.3(NP)	2.8–6.7	4.8	1.9–7.3	4.6	1.5–8.6	5.0	2.1–8.3	5.2
β-CTLN	1.4–6.1	2.7(NP)	0.9–4.5	2.7	1.0–4.5	2.7	0.9–4.5	2.7	1.3–4.8	2.5(NP)	0.9–4.1	2.5
α-cortol	0.5–1.7	0.8(NP)	0.5–1.2	0.7(NP)	0.5–1.2	0.9	0.4–1.6	0.8(NP)	0.2–1.8	1.0	0.3–2.1	1.2
Progesterone												
aP2	0.2–1.3	0.4(NP)	0.1–0.9	0.4(NP)	0.1–0.7	0.4	0.0–0.6	0.3	0.0–0.6	0.2(NP)	0.0–0.5	0.2(NP)
P2	0.6–3.7	1.3(NP)	0.3–2.4	1.0(NP)	0.5–2.2	1.0(NP)	0.3–2.6	0.8(NP)	0.2–2.4	0.8(NP)	0.1–2.0	1.1
P3	2.2–8.4	4.3(NP)	0.6–6.0	3.3	1.2–5.3	3.3	1.4–6.4	2.8(NP)	1.0–6.5	2.2(NP)	1.3–5.2	2.6(NP)
Polone	0.0–0.7	0.2(NP)	0.0–0.3	0.2(NP)	0.0–0.3	0.2(NP)	0.0–0.3	0.1(NP)	0.0–0.3	0.1(NP)	0.0–0.3	0.1(NP)
Aldosterone												
THA	0.1–1.1	0.6	0.1–1.1	0.4(NP)	0.1–0.8	0.4	0.1–0.9	0.5	0.0–1.1	0.4(NP)	0.1–0.9	0.5
THB	0.3–1.4	0.6(NP)	0.1–1.1	0.5	0.2–1.1	0.5(NP)	0.2–1.3	0.5(NP)	0.1–1.7	0.56(NP)	0.2–1.3	0.7
aTHB	0.3–3.3	1.8	0.1–3.4	1.7	0.3–2.7	1.5	0.6–3.2	1.3(NP)	0.3–3.4	1.0(NP)	0.1–3.0	1.6

Table 3: Continued

Steroid metabolite	Age decade, years											
	20–29		30–39		40–49		50–59		60–69		70–79	
	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]
Intermediate												
THS	0.0–0.6	0.2$^{(NP)}$	0.0–0.4	0.2$^{(NP)}$	0.0–0.3	0.2	0.0–0.5	0.2	0.1–0.7	0.3$^{(NP)}$	0.1–0.7	0.3$^{(NP)}$
PDL	0.4–2.4	0.9$^{(NP)}$	0.3–2.0	0.8$^{(NP)}$	0.4–1.8	0.8$^{(NP)}$	0.2–2.0	0.9$^{(NP)}$	0.3–1.9	0.7$^{(NP)}$	0.2–1.6	0.9
PTL	<0.1	<0.1	<0.1	<0.1	0.0–0.2	0.05$^{(NP)}$	0.0–0.2	0.06$^{(NP)}$	0.0–0.4	0.06$^{(NP)}$	0.0–0.2	0.07$^{(NP)}$
aPDL	0.0–0.4	0.2$^{(NP)}$	0.0–0.3	0.1$^{(NP)}$	0.0–0.3	0.1$^{(NP)}$	0.0–0.3	0.1$^{(NP)}$	0.0–0.2	0.1$^{(NP)}$	0.0–0.2	0.1$^{(NP)}$
TH-DOC	≤0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	≤0.1	<0.1	<0.1	<0.1
Fetal												
A'3	1.0–9.6	2.5$^{(NP)}$	0.5–9.9	2.2$^{(NP)}$	0.5–5.1	2.1$^{(NP)}$	0.5–4.4	1.4$^{(NP)}$	0.1–3.0	1.3	0.5–3.4	1.2$^{(NP)}$
16K-A'2	0.2–1.5	0.5$^{(NP)}$	0.1–1.5	0.4$^{(NP)}$	0.0–0.7	0.3	0.0–0.8	0.3$^{(NP)}$	0.0–0.8	0.2$^{(NP)}$	0.0–0.8	0.3$^{(NP)}$
16-OH-DHEA	1.2–11.3	4.2$^{(NP)}$	0.2–12.6	2.0$^{(NP)}$	0.4–3.4	1.9	0.1–4.2	1.2$^{(NP)}$	0.1–3.2	0.7$^{(NP)}$	0.2–4.6	1.0$^{(NP)}$
16,18-$(OH)_2$-DHEA	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
16-OH-p'ol	0.0–0.4	0.2	0.0–0.4	0.1$^{(NP)}$	0.0–0.2	0.1$^{(NP)}$	0.0–0.2	0.1$^{(NP)}$	<0.1	<0.1	0.0–0.2	<0.1
15-OH-PDL	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
17-P3	0.1–4.0	2.0	0.2–3.9	1.1$^{(NP)}$	0.2–2.3	1.2	0.1–1.7	0.8	0.1–1.3	0.5$^{(NP)}$	0.1–1.5	0.8
15-OH-P'DL	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

[a]Data are presented as mean for parametric data or median for non-parametric (NP) data. Abbreviations as in Figure 1 and Supplemental Table 1.

Reference intervals and influence of OCP

As shown in Tables 3 and 4, almost all steroid excretions were different between the various age and sex groups. Androgen metabolite excretion declined with age in men and women, with absolute concentrations much higher in men compared to women (Figures 2 and 3). Cortisol metabolite excretion remained constant during life in both sexes but increased in women 70–79 years of age. Progesterone metabolite excretion peaked in 30–39 year old women and declined afterwards. Fetal metabolites declined in both sexes during life. In addition to absolute reference intervals we also calculated age and sex specific reference intervals for diagnostic ratios, as presented in Supplementary Table 4.

Figure 2: Median sum scores of urinary steroid metabolite excretion in men per decade (n = 120).

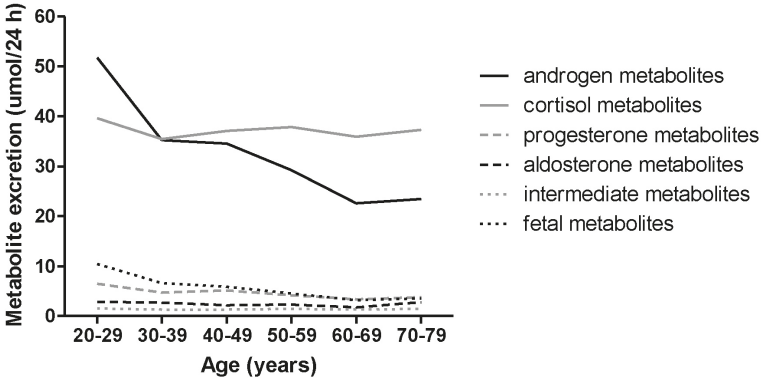
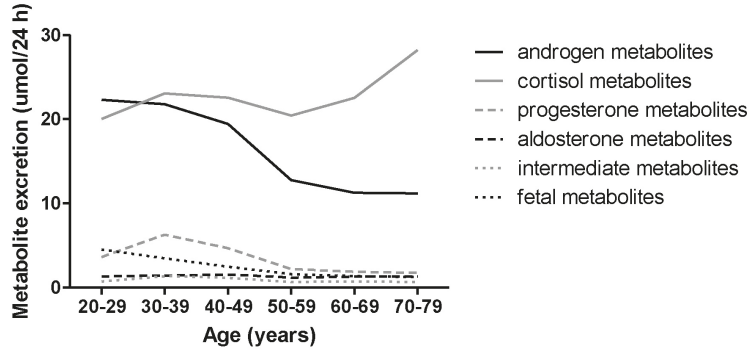
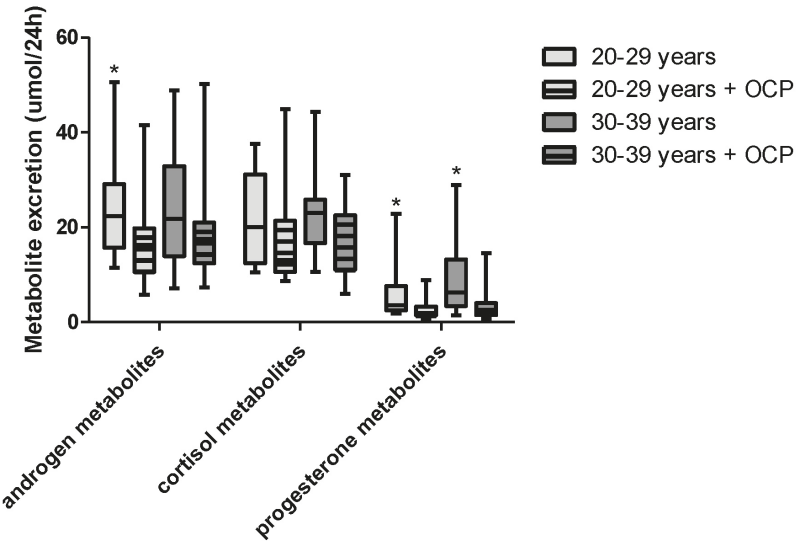


Figure 3: Median sum scores of urinary steroid metabolite excretion in women not using OCP per decade.



There were significant differences between distributions of metabolite excretions for women with or without the use of OCP (Table 4 and Figure 4). Women using OCP had lower excretions of androgen metabolites in the 20–29 year old age group (16.3 [10.6–19.8] vs. 21.8 [13.9–23.9] μmol/24 h, p = 0.042). Progesterone metabolite excretion was decreased in both age groups using OCP compared to corresponding age groups not using OCP (20–29 years: 1.9 [1.5–3.3] vs. 3.6 [2.5–7.6] μmol/24 h, p = 0.006 and 30–39 years: 2.6 [1.5–4.0] vs. 6.3 [3.4–13.2] μmol/24 h, p = 0.001). Multiple cortisol metabolites were excreted in significantly lower amounts in the women using OCP (Table 4). Total cortisol metabolite excretion showed a trend toward lower excretion in women using OCP in both age groups (20–29 years: 13.1 [10.6–21.4] vs. 20.0 [12.4–31.2] μmol/24 h, p = 0.058 and 30–39 years: 15.8 [11.1–22.5] μmol/24 h, p = 0.055, Figure 4).

Figure 4: Urinary metabolite excretion in women with versus without use of OCP.



Boxes represent median with interquartile range. Whiskers represent minimum and maximum values. OCP, oral contraceptive pills. *p < 0.05 compared to the same age group using OCP.

Table 4: Reference intervals of urinary steroid metabolites in women (μmol/24 h) per decade.

Steroid metabolite	Age decade, years															
	20–29						30–39		40–49		50–59		60–69		70–79	
	OCP -		OCP +		OCP -		OCP +									
	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]
Androgen																
A	2.8–20.8	6.7(NP)	0.9–12.2	6.5	0.1–17.3	8.0	1.9–10.9b	6.4	2.1–15.4	5.2(NP)	1.1–7.0	2.7(NP)	0.5–8.8	2.5(NP)	0.3–6.0	1.9(NP)
E	1.8–14.4	8.1	1.1–10.4	5.8	1.4–13.2	7.3	1.9–17.8	6.9(NP)	2.6–17.7	6.2(NP)	0.9–7.4	4.2	1.2–9.7	3.3(NP)	0.8–7.6	2.5(NP)
DHEA	0.2–11.8	1.2(NP)	0.1–2.5b	0.7(NP)	0.1–12.2	0.8(NP)	0.2–3.4	0.9(NP)	0.1–5.7	0.7(NP)	0.2–1.0	0.3(NP)	0.1–0.8	0.3(NP)	0.0–0.6	0.2(NP)
11-KE	0.4–3.1	1.0(NP)	0.1–2.3c	0.5(NP)	0.5–2.8	1.1(NP)	0.2–3.1c	0.8(NP)	0.1–3.1	1.6	0.6–3.9	1.4(NP)	0.4–3.6	1.5(NP)	0.5–3.8	1.9
11-HA	0.6–6.3	1.7(NP)	0.5–3.1	1.0(NP)	0.6–6.0	2.2(NP)	0.5–3.7c	1.5(NP)	0.6–5.2	2.9	1.0–5.3	2.3(NP)	0.3–4.8	2.5	0.3–5.0	2.8
11-HE	0.1–2.3	1.2	0.0–2.2b	0.6(NP)	0.3–3.8	1.3(NP)	0.1–3.3	0.8(NP)	0.2–3.1	1.6	0.2–3.0	1.6	0.1–2.1	1.0	0.4–4.7	1.4(NP)
Estriol	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.0–0.3	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Cortisol																
THE	3.1–23.4	7.8(NP)	2.3–11.2c	4.7(NP)	2.6–14.4	8.5	1.3–15.1c	5.3(NP)	4.7–18.2	9.2(NP)	4.5–18.4	8.3(NP)	4.8–19.4	9.1(NP)	3.4–18.8	11.1
THF	1.0–7.1	4.1	1.6–5.6	3.0(NP)	2.2–8.7	4.2(NP)	0.4–7.4	3.9	2.8–8.0	4.5(NP)	1.8–7.7	4.7	2.6–12.7	5.4(NP)	2.2–10.0	6.1
aTHF	0.4–10.4	3.2(NP)	0.4–5.0c	1.3(NP)	0.1–7.6	3.6	0.7–4.2c	1.8(NP)	0.9–5.4	3.1	1.1–6.3	2.4(NP)	0.7–4.8	2.8	0.5–9.0	2.3(NP)
α-CTLN	0.9–7.4	4.1	1.1–5.6	3.4	1.5–7.4	3.3(NP)	1.3–5.4	3.3	1.3–6.6	3.9	0.9–6.4	3.7	2.1–7.1	3.5(NP)	1.8–7.5	4.7
β-CTLN	0.1–3.3	1.7	0.4–2.4b	0.9(NP)	0.4–2.8	1.6	0.4–2.4c	1.2	0.7–4.0	1.6(NP)	0.8–3.2	1.5(NP)	0.8–3.1	1.6(NP)	0.7–3.5	2.1
α-cortol	0.1–1.1	0.6	0.3–1.0	0.6	0.3–1.3	0.5	0.3–1.5	0.7(NP)	0.3–1.0	0.6	0.3–1.2	0.5(NP)	0.3–1.4	0.7(NP)	0.3–1.4	0.9
Progesterone																
aP2	0.0–3.5	0.3(NP)	0.0–0.4	0.2(NP)	0.0–3.6	0.5(NP)	0.0–0.4c	0.1(NP)	0.1–5.6	0.3(NP)	0.0–0.3	0.2(NP)	0.0–0.4	0.1(NP)	0.0–0.2	0.1(NP)
P2	0.3–14.2	1.5(NP)	0.2–2.1b	0.7(NP)	0.4–21.9	2.2(NP)	0.2–4.4c	1.0(NP)	0.2–25.6	1.6(NP)	0.2–1.4	0.6(NP)	0.2–1.7	0.7(NP)	0.1–1.2	0.6
P3	0.6–5.7	2.1(NP)	0.3–2.9b	0.8(NP)	0.7–6.5	2.7(NP)	0.3–5.6c	1.4(NP)	0.1–5.3	2.6	0.5–2.6	1.1(NP)	0.5–2.7	1.0(NP)	0.2–1.8	1.0
Polone	0.0–1.3	0.2(NP)	0.0–0.3c	0.1(NP)	0.0–2.2	0.2(NP)	0.0–0.4b	0.1(NP)	0.0–2.5	0.2(NP)	0.0–0.2	0.1(NP)	0.0–0.2	0.1(NP)	<0.1	<0.1
Aldosterone																
THA	0.1–1.3	0.3(NP)	0.1–0.6	0.3(NP)	0.0–0.5	0.3	0.0–0.8	0.2(NP)	0.2–0.6	0.3(NP)	0.1–0.8	0.3(NP)	0.1–0.9	0.3(NP)	0.0–0.8	0.3(NP)
THB	0.1–1.3	0.4(NP)	0.1–0.7	0.3(NP)	0.1–0.8	0.4(NP)	0.1–1.0	0.3(NP)	0.2–0.7	0.4	0.2–0.9	0.4(NP)	0.1–1.3	0.4(NP)	0.2–1.0	0.4(NP)
aTHB	0.2–3.5	0.8(NP)	0.2–1.5b	0.4(NP)	0.1–1.5	0.8	0.2–1.6b	0.5(NP)	0.2–1.3	0.8	0.2–1.5	0.5(NP)	0.2–1.0	0.6	0.2–1.7	0.6(NP)
Intermediate																
THS	0.0–0.3	0.1(NP)	0.0–0.3	0.1(NP)	0.0–0.3	0.2(NP)	0.0–0.3	0.1(NP)	0.1–0.4	0.2(NP)	0.0–0.6	0.2(NP)	0.1–0.7	0.2(NP)	0.0–0.6	0.2(NP)
PDL	0.1–1.9	0.4(NP)	0.0–0.6b	0.2(NP)	0.1–2.1	0.9	0.0–1.0c	0.2(NP)	0.1–2.6	0.6(NP)	0.0–0.7	0.2(NP)	0.0–0.6	0.2(NP)	0.0–0.4	0.2(NP)
PTL	0.0–0.2	0.04(NP)	<0.1c	<0.1	0.0–0.2	0.03(NP)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.0–0.4	0.06(NP)	0.0–0.3	0.05(NP)
aPDL	0.0–0.2	0.1(NP)	<0.1	<0.1	0.0–0.2	<0.1	<0.1	<0.1	0.0–0.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
TH-DOC	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Fetal																
A'3	0.3–5.8	1.1(NP)	0.1–2.4	1.2	0.2–2.8	1.1(NP)	0.3–4.0	1.0(NP)	0.3–2.4	0.9(NP)	0.1–1.1	0.6	0.1–1.9	0.5(NP)	0.0–0.8	0.4
16K-A'2	0.0–1.2	0.3(NP)	0.0–0.4	0.1(NP)	0.0–0.6	0.2(NP)	0.0–0.7	0.2(NP)	0.0–0.5	0.2(NP)	0.0–0.3	0.1(NP)	0.0–0.2	0.1(NP)	0.0–0.2	0.1(NP)
16-OH-DHEA	0.1–3.4	1.7	0.1–2.2	1.0	0.1–5.0	1.0(NP)	0.2–4.4	0.9(NP)	0.2–3.3	0.6(NP)	0.0–1.5	0.3(NP)	0.0–1.1	0.2(NP)	0.0–0.5	0.2(NP)
16,18-$(OH)_2$-DHEA	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
16-OH-p'ol	0.0–0.2	<0.1	0.0–0.2	<0.1	<0.1	<0.1	0.0–0.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
15-OH-PDL	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
17-P3	0.1–2.2	0.8(NP)	0.0–1.1c	0.3(NP)	0.0–2.8	0.5(NP)	0.0–2.1	0.3(NP)	0.0–1.1	0.3(NP)	0.0–0.7	0.2(NP)	0.0–0.4	0.2(NP)	0.0–0.4	0.1(NP)
15-OH-P'DL	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

[a]Data are presented as mean for parametric data or median for non-parametric (NP) data. [b] $p < 0.1$ compared to women of the same age not taking OCP. [c] $p < 0.05$ compared to women of the same age not taking OCP. Abbreviations as in Figure 1 and Supplemental Table 1.

Discussion

To the best of our knowledge, we here describe for the first time age- and sex-specific reference intervals for urinary steroid metabolite excretion in a well-defined and large healthy adult population with a broad age range. These urinary steroid profiles were determined with a newly-developed and validated GC-MS/MS assay.

Well-defined reference intervals for the adult population are essential, as USP in adult subjects has proven to be a valuable diagnostic tool in several clinical situations. For example, congenital adrenal hyperplasia (CAH), in particular the milder variants, might be diagnosed for the first time in adulthood (6,7) by noticing moderately elevated P3, PTL and PDL excretions and/or elevated diagnostic ratios for these enzyme deficiencies (Supplementary Table 4). In addition, USP can be helpful to evaluate the efficacy of glucocorticoid treatment in adult patients with CAH or for the diagnosis of licorice induced hypertension, which is reflected by an impaired hydroxysteroid dehydrogenase activity (8). Patients with ACC often demonstrate several disorders in steroid biosynthesis. Retrospective data demonstrate the diagnostic potential of USP to differentiate between a benign adenoma and ACC (10, 11). These abnormalities in steroid biosynthesis are clinically useful for both diagnosis as well as for the follow-up of patients with ACC (9).

Our data show that excretions of almost all 33 analyzed steroid metabolites are affected by age and gender, underscoring the need for age and sex specific reference intervals (Tables 3 and 4). In addition, it was demonstrated that use of OCP resulted in a significantly lower urinary excretion of progesterone and androgen metabolites. The decrease in progesterone and androgen excretion can be explained by an OCP induced inhibition of the hypothalamic-pituitary-gonadal axis. In addition, there was a trend for a lower urinary cortisol metabolite excretion in women on OCP, which is likely to result from an OCP induced elevation of the plasma cortisol-binding globulin (CBG) concentration (20, 21).

Our data are in agreement with previous reports showing a decline in androgen production with age, while cortisol production remains relatively stable throughout adult life (Figures 2 and 3) (16, 22, 23). The observed increase in cortisol metabolite excretion among women aged 70–79 years might be due to a decline of CBG levels or differences in body composition (24). In addition, a survivor effect cannot be excluded. The decrease in progesterone metabolite excretion in women older than 50 years of age most likely reflects their postmenopausal status. Importantly,

reference intervals for the diagnostic ratios of disorders in steroid biosynthesis are also significantly affected by age and sex (Supplementary Table 4). Calculation of these diagnostic ratios is very helpful in clinical practice for the detection of various inborn errors of steroid biosynthesis and the follow-up of these conditions throughout adulthood (4, 6, 14). Reference intervals for urinary steroid metabolites have been reported in several previous studies (4,6,11,13–17). We believe our study design is the most optimal one currently available. Previous published reference intervals did not take the age dependency of metabolite excretion into account as demonstrated in the current study. It is therefore reasonable that our reference intervals differ slightly compared to studies in which the population was analyzed as a whole irrespective of age. Obviously, the latter approach results in regression to the mean. Also, analytically we optimized the setting by applying isotope labeled internal standards as much as possible, using the most specific technique currently available. Moreover, the strength of our study is the selection of a large healthy population. Consequently, we believe that our reference intervals are useful in an adult population for anyone using a similar GC-MS/MS (or GC-MS) method to ours.

The here described newly-developed GC-MS/MS assay has several advantages over our previous GC-MS method. First of all, GC-MS/MS methods have higher specificity than GC-MS methods. Tandem mass spectrometry has the ability to select one mass-to-charge ratio (*m/z*) ion and create specific fragmented ions out of this precursor ion in the second mass spectrometer. Consequently, interference by other analytes with the same precursor *m/z*, but different product ions, is avoided. In addition, the GC-MS/ MS equipment provides an improved baseline separation, which enables automatic peak integration and reduces time of analysis (25). Pre-set requirements (based on ISO15189 and Dutch guidelines for validation of analytical methods) for precision, linearity, recovery, LLOQ, carry-over and stability were met for all steroid metabolites and results were improved compared to the former GC-MS method. Only for 16-KA'2 the inter- and intra-assay CVs were noted to be relatively high. This particular steroid metabolite, however, is of limited diagnostic value. Another advantage of the GC-MS/MS is the reduction of analysis time with 1 day. In addition, the integrated software provides automatic chromatographic data integration, whereby obtained results are transferred to the laboratory information system, which in turn generates user-friendly graphical data reports.

For more than a decade we and 27 other laboratories from all over the world participate in an external steroid profile quality assessment scheme organized

by the University College London Hospitals (London, UK) and the Stichting Kwaliteitsbewaking Medische Laboratorium diagnostiek (SKML, The Netherlands). Since a standard reference method is lacking, participation guarantees quality and is the best way of demonstrating accuracy. In this quality scheme our GC-MS/MS method performs better than average and scores in the top 7 (range 1–7) of 28 laboratories in the year 2016 with a constant MOM score of 2 or higher.

Although liquid chromatography-tandem mass spectrometry (LC/MS-MS) is expected to become the high- throughput method of choice for targeted limited steroid determinations, we agree with other authors (4) that USP by GC-MS will remain the most powerful discovery tool for defining disorders of steroid biosynthesis for years to come. Advantages of applying USP instead of targeted blood measurements of adrenal steroids are the elimination of diurnal variation by using 24 h urine collections and the generation of a so-called metabolome including characterization of unknown metabolites. LC-MS/MS is not capable of profiling >20–30 metabolites simultaneously, although a method for glucocorticoid metabolites has been described (26). For such profiling chromatography with high plate numbers remains very important and therefore GC-MS is still the superior technique. A future development could be the use of a multidimensional statistical approach of data interpretation (metabolomics). Data from the current study might be very useful for the development of such a tool.

In conclusion, we developed a new GC-MS/MS method for USP which is suitable for high-throughput analysis. Widely applicable age and sex specific reference intervals for 33 metabolites and their diagnostic ratios have been defined for male and female individuals aged 20–79 years. In addition, we have shown that the use of OCP influences USP in women 20–39 years of age, which should be taken into account when interpreting these results.

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Supplemental data

Supplemental Table 1: Steroid abbreviations and nomenclature according to LOINC and ADC/ IUPAC including Chemspider ID.

LOINC nomenclature (Trivial name)	Manuscript abbreviation	ACD/IUPAC Name	Chemspider ID
Androsterone	A	(3α,5α)-3-Hydroxyandrostan-17-one	5668
Etiocholanolone	E	(3α,5β)-3-Hydroxyandrostan-17-one	5669
Dehydroepiandrosterone	DHEA	(3β)-3-Hydroxyandrost-5-en-17-one	5670
11-Keto-etiocholanolone	11-KE	(3α,5β)-3-Hydroxyandrostane-11,17- dione	92021
11-Hydroxyandrosterone	11-HA	(3α,5α,11ß)-3,11-Dihydroxyandrostan-17-one	8461834
11-Hydroxyetiocholanolone	11-HE	(3α,5β,11β)-3,11-Dihydroxyandrostan- 17-one	92020
Tetrahydrocortisone	THE	(3α,5β)-3,17,21-Trihydroxypregnane- 11,20-dione	5657
Tetrahydrocortisol	THF	(3α,5β,11β)-3,11,17,21- Tetrahydroxypregnan-20-one	5655
Allo-tetrahydrocortisol	aTHF	(3α,5α,11β)-3,11,17,21-Tetrahydroxypregnan-20-one	83726
Alpha cortolone	α-CTLN	(3α,5β,20S)-3,17,20,21-Tetrahydroxypregnan-11-one	141038
Beta cortolone	β-CTLN	(3α,5β,20R)-3,17,20,21-Tetrahydroxypregnan-11-one	214529
Alpha-cortol	α-cortol	(3α,5β,11β,20S)-Pregnane-3,11,17,20,21-pentol	216069
Allo-pregnanediol	aP2	(3α,5α,20S)-Pregnane-3,20-diol	144360
Pregnanediol	P2	(3α,5β,20S)-Pregnane-3,20-diol	190585
Pregnanetriol	P3	(3α,5β,20S)-Pregnane-3,17,20-triol	92121
Epipregnanolone	Polone	(3β,5β)-3-Hydroxypregnan-20-one	198867
Estriol	Estriol	(16α,17β)-Estra-1,3,5(10)-triene- 3,16,17-triol	5553
11-Dehydrotetrahydro- corticosterone	THA	(3α,5β)-3,21-Dihydroxypregnane- 11,20-dione	194305
Tetrahydrocorticosterone	THB	3,11,21-Trihydroxypregnan-20-one	10145
Allo- tetrahydrocorticosterone	aTHB	(3α,5α,11β)-3,11,21-Trihydroxypregnan-20-one	91970
Tetrahydrodeoxycortisol	THS	(3α,5β)-3,17,21-Trihydroxypregnan-20-one	58998
Pregnanediolone	PDL	(3α,5β)-3,17-Dihydroxypregnan-20- one	91955

Supplemental Table 1: Continued

LOINC nomenclature (Trivial name)	Manuscript abbreviation	ACD/IUPAC Name	Chemspider ID
Pregnanetriolone	PTL	(3α,5β,20S)-3,17,20-Trihydroxypregnan-11-one	223202
Allo-pregnanediolone	aPDL	(3α,5α)-3,17-Dihydroxypregnan-20- one	99828
11-Deoxytetrahydro-corticosterone	TH-DOC	(3α,5α)-3,21-Dihydroxypregnan-20-one	91953
Androstenetriol	A'3	(3β,16α,17β)-Androst-5-ene-3,16,17-triol	7993771
16-Ketoandrostenediol	16K-A'2	(3β,17β)-3,17-Dihydroxyandrost-5-en-16-one	132993
16-Alpha hydroxydehydroepi-androsterone	16-OH- DHEA	(3β,16α)-Androst-5-ene-3,16-diol	18185
16 Beta,18- dihydroxydehydroepi-androsterone	16,18-$(OH)_2$- DHEA	(3β,16α)-Androst-5-ene-3,16,18-triol (not available)	-
16-Alpha hydroxypregnenolone	16-OH-P'OL	(3β,16α)-3,16-Dihydroxypregn-5-en- 20-one	4955002
3-Alpha, 15-beta, 17 alpha-trihydroxypregnanediolone	15-OH-PDL	(3α,5β,15β)-3,15,17-Trihydroxypregnan-20-one	117727
17-Hydroxypregnenetriol	17-P3	(3β,20S)-Pregn-5-ene-3,17,20-triol	132969
15-Hydroxypregnenolone	15-OH-P'DL	(3β,15β)-3,15,17-Trihydroxypregn-5- en-20-one	117725

Supplemental Table 2: Recovery ranges (n=6) obtained by spiking 3 different urine samples with 3 concentrations of standard solution.

	Urine sample 1			Urine Sample 2			Urine sample 3		
Component	Spike 25 µL	Spike 50 µL	Spike 75 µL	Spike 25 µL	Spike 50 µL	Spike 75 µL	Spike 25 µL	Spike 50 µL	Spike 75 µL
A	96 – 100	95 – 105	99 - 104	98 - 106	100- 105	100- 103	95 - 103	96 - 105	94 - 103
E	95 – 98	96 – 103	99 - 104	97 - 106	99 - 107	99 - 105	93 - 99	96 - 105	94 - 107
DHEA	90 – 100	93 – 99	94 - 99	92 - 98	96 - 100	94 - 98	91 - 98	94 - 98	95 - 97
11-KE	93 – 105	92 – 101	95 - 101	92 - 100	96 - 100	95 - 100	90 - 98	95 - 100	93 - 99
PDL	93 – 105	93 – 110	99 - 106	93 - 109	98 - 108	96 - 108	95 - 109	98 - 110	97 - 106
11-HA	92 – 102	94 – 105	98 - 103	93 - 107	101- 106	99 - 103	92 - 104	94 - 101	94 - 102
11-HE	91 – 103	91 – 103	97 - 104	92 - 106	98 - 102	99 - 102	91 - 104	94 - 102	94 - 100
polone	95 – 104	95 – 106	98 - 105	94 - 104	96 - 107	97 - 106	91 - 106	95 - 107	96 - 106
allo-PDL	94 – 101	92 – 108	97 - 106	91 - 103	98 - 104	94 - 104	92 - 105	97 - 103	95 -104
16-OH- DHEA	91 – 107	96 – 106	95 - 101	92 - 97	96 - 102	94 - 106	90 - 97	99 - 105	96 - 106
allo-P2	94 – 101	93 – 107	96 - 105	94 - 103	97 - 102	96 - 109	91 - 102	95 - 102	96 - 100
P2	94 – 103	91 – 105	96 - 104	95 - 103	97 - 101	96 - 110	94 - 106	99 - 106	97 - 103
P3	94 – 102	94 - 105	98 - 104	93 - 103	97 - 103	97 - 108	92 - 102	95 - 103	94 - 101
16-KA'2	89 – 94	96 - 99	99 - 105	92 - 104	100- 101	100- 105	91 - 99	96 - 102	96 - 102
A3	93 – 100	94 - 107	98 - 107	95 - 106	99 - 106	98 - 112	92 - 102	99 - 110	97 - 106
THS	94 – 103	94 - 105	100- 106	94 - 105	99 - 104	98 - 105	93 - 106	99 - 106	96 - 103
Oestriol	94 – 108	94 - 108	98 - 104	93 - 107	98 - 106	98 - 110	92 - 106	100-108	100-106
TH-DOC	92 – 105	96 - 106	100- 107	92 - 105	99 - 105	99 - 104	91 - 107	101-105	99 - 103
PTL	92 – 102	92 - 107	97 - 104	91 - 105	98 - 105	96 - 109	91 - 102	97 - 108	95 - 103
16-OH-p'ol	93 – 101	98 - 104	95 - 103	94 - 103	99 - 103	98 - 104	93 - 102	98 - 105	96 - 101
17-P3	92 – 103	91 - 106	97 - 104	93 - 105	98 - 104	97 - 108	90 - 103	95 - 104	94 - 101
THE	93 – 102	94 - 99	95 - 102	97 - 109	94 - 109	96 - 107	94 - 103	96 - 105	94 - 101
THA	89 – 104	96 - 103	98 - 105	92 - 102	99 - 103	102- 106	92 - 99	99 - 102	98 - 103
THB	95 – 102	97 - 105	102- 106	93 - 103	102- 106	97 - 106	91 - 102	100-105	100-105
allo-THB	93 – 105	98 - 108	100- 106	93 - 105	101- 107	97 - 104	92 - 105	99 - 104	97 - 106
THF	97 – 111	98 - 105	97 - 104	97 - 114	100- 110	100- 104	91 - 109	97 - 106	96 - 102
allo-THF	97 – 109	96 - 109	98 - 102	94 - 106	100- 106	96 - 103	94 - 106	99 - 105	96 - 103
α-CTLN	94 – 107	96 - 106	99 - 103	95 - 110	99 - 109	100- 106	91 - 101	97 - 108	95 - 102
β-CTLN	95 – 107	96 - 106	99 - 104	95 - 105	100- 105	101- 107	92 - 104	98 - 109	97 - 103
α-cortol	94 – 108	95 - 107	98 - 106	95 - 110	100- 106	98 - 109	92 - 103	98 - 107	95 - 104

Abbreviations as in Figure 1 and Supplemental Table 1.

Supplemental Table 3: Method comparison of GC-MS with GC-MS/MS

Steroid metabolites	Slope	Intercept
Androgen		
A	1.17*	0.01
E	0.99	0.04
DHEA	0.98*	0.02#
11-KE	1.12*	0.02#
11-HA	0.11	1.19#
11-HE	0.07*	1.21#
Cortisol		
THE	0.00	1.01
THF	0.12*	1.13#
aTHF	-0.03	1.33#
α-CTLN	0.03	1.01
β-CTLN	0.02	1.02
α-cortol	-0.09	1.97#
Progesterone		
aP2	0.00	1.34#
P2	-0.02*	1.13#
P3	0.02	1.20#
Polone	-0.01*	0.98
Aldosterone		
THA	0.01	1.42#
THB	0.06*	1.08
aTHB	0.00	1.45#
Intermediate		
THS	0.01	1.07
PDL	0.00	1.19#
PTL	0.00	1.14#
aPDL	0.01	1.29#
Fetal		
A'3	0.00	1.12#
16K-A'2	0.01	1.03
16-OH-DHEA	0.03*	0.92
16-OH-p'ol	0.00	1.08
17-P3	0.00	1.13#

*: slope significantly different from 1.00 (P<0.05) # intercept significantly different from 0.00 (P<0.05)

Abbreviations as in Figure 1 and Supplemental Table 1.

Supplemental Table 4: Reference intervals for diagnostic ratios of disorders in steroidogenesis in men and women per decade.

Steroid disorder	Ratio	Male						Female							
								20-29		30-39		Age decade, years			
		20-29	30-39	40-49	50-59	60-69	70-79	OCP-	OCP+	OCP-	OCP+	40-49	50-59	60-69	70-79
3β-HSD deficiency	DHEA/(THE+THF+aTHF)	0.0-0.8	0.0-0.6	0.0-0.4	0.0-0.2	0.0-0.1	0.0-0.2	0.0-0.7	0.0-0.3	0.0-0.5	0.0-0.4	0.0-0.4	0.0-0.1	0.0-0.04	0.0-0.0
	(A+E)/(THE+THF+aTHF)	0.5-1.9	0.3-1.4	0.4-1.2	0.2-1.1	0.2-0.9	0.2-1.0	0.1-2.2	0.2-2.4	0.2-1.7	0.4-2.2	0.3-1.7	0.2-0.9	0.1-0.8	0.0-0.
5α-reductase deficiency	E/A	0.3-1.8	0.1-1.1	0.4-1.2	0.3-1.4	0.5-1.5	0.5-1.9	0.3-1.8	0.4-1.9	0.2-2.1	0.5-2.2	0.6-2.4	0.5-2.5	0.6-4.3	0.5-2.
	THF/aTHF	0.3-2.8	0.4-1.8	0.4-1.5	0.5-1.8	0.6-2.7	0.7-3.1	0.5-4.1	0.3-4.2	0.5-3.7	0.5-3.8	0.7-4.1	0.7-4.2	0.7-7.4	0.7-6.
	THB/aTHB	0.1-1.2	0.2-0.6	0.2-0.6	0.2-0.7	0.2-1.0	0.2-1.0	0.2-1.5	0.1-1.2	0.2-1.4	0.1-1.2	0.1-1.1	0.3-1.6	0.2-2.4	0.2-2.
11β-HSD 2 deficiency / 11β-HSD 1 deficiency	(THF+aTHF)/THE	0.5-2.4	0.7-1.6	0.7-1.8	0.6-1.7	0.5-1.7	0.7-1.8	0.4-1.2	0.6-1.5	0.4-1.6	0.5-1.6	0.5-1.2	0.5-1.1	0.5-1.3	0.5-1.
17β-HSD deficiency	(A+E)/(THE+THF+aTHF)	0.5-1.9	0.3-1.4	0.4-1.2	0.2-1.1	0.2-0.9	0.2-1.0	0.1-2.2	0.2-2.4	0.2-1.7	0.4-2.2	0.3-1.7	0.2-0.9	0.1-0.9	0.0-0.
P450 oxidoreductase deficiency (POR)	(PDL+P3)/(A+E)	0.1-0.2	0.1-0.3	0.1-0.3	0.1-0.4	0.1-0.4	0.1-0.4	0.1-0.4	0.0-0.2	0.1-0.9	0.1-0.3	0.1-0.7	0.1-0.5	0.1-0.6	0.1-0.
	(PDL+P3)/(THE+THF+aTHF)	0.1-0.3	0.1-0.3	0.1-0.2	0.1-0.3	0.1-0.3	0.1-0.2	0.0-0.3	0.1-0.3	0.1-0.4	0.1-0.3	0.0-0.4	0.0-0.2	0.0-0.2	0.0-0.
	P2/(THE+THF+aTHF)	0.0-0.1	0.0-0.1	0.0-0.1	0.0-0.1	0.0-0.1	0.0-0.1	0.0-0.8	0.0-0.2	0.0-1.2	0.0-0.3	0.0-1.3	0.0-0.1	0.0-0.1	0.0-0.
11β-hydroxylase deficiency	THS/(THE+THF+aTHF)	0.0-0.02	0.0-0.02	0.0-0.02	0.0-0.02	0.0-0.02	0.0-0.02	0.0-0.01	0.0-0.03	0.0-0.02	0.0-0.03	0.0-0.02	0.0-0.02	0.0-0.03	0.0-0.0
17α-hydroxylase deficiency	(THA+THB+aTHB)/(THE+T HF+aTHF)	0.0-0.1	0.0-0.2	0.0-0.2	0.0-0.1	0.0-0.1	0.0-0.1	0.1-0.2	0.1-0.2	0.0-0.2	0.1-0.2	0.0-0.2	0.0-0.1	0.0-0.1	0.0-0.
	(THA+THB+aTHB)/(A+E)	0.0-0.2	0.0-0.3	0.1-0.2	0.1-0.3	0.0-0.3	0.1-0.5	0.0-0.4	0.0-0.3	0.0-0.3	0.0-0.2	0.0-0.3	0.1-0.5	0.1-0.9	0.1-0.
21-hydroxylase deficiency	PDL/(THE+THF+aTHF)	0.0-0.1	0.0-0.1	0.0-0.1	0.0-0.1	0.0-0.1	0.0-0.1	0.0-0.1	0.0-0.05	0.0-0.1	0.0-0.1	0.0-0.1	0.0-0.03	0.0-0.02	0.0-0.0
	P3/(THE+THF+aTHF)	0.1-0.3	0.1-0.3	0.1-0.2	0.0-0.2	0.0-0.2	0.0-0.2	0.0-0.3	0.0-0.2	0.0-0.3	0.1-0.3	0.0-0.3	0.0-0.2	0.0-0.1	0.0-0.
	PTL/(THE+THF+aTHF)	0.0-0.004	0.0-0.004	0.0-0.004	0.0-0.007	0.0-0.01	0.0-0.01	0.0-0.02	0.0-0.004	0.0-0.01	0.0-0.01	0.0-0.01	0.0-0.005	0.0-0.02	0.0-0.0

Abbreviations as in Figure 1 and Supplemental Table 1.

Chapter 3

High-density lipoproteins and adrenal steroidogenesis: A population-based study

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Abstract

Background: Cholesterol trafficked within plasma lipoproteins, in particular high-density lipoproteins (HDL), may represent an important source of cholesterol that is required for adrenal steroidogenesis. Based on a urinary gas chromatography method, compromised adrenal function has been suggested in men but not in women with (genetically determined) low plasma HDL-cholesterol (HDL-C).

Objective: The objective of the article was to examine the extent to which glucocorticoid production relates to HDL-C in a population-based cohort.

Methods: A total of 240 subjects (120 men and 120 women, aged 20–79 years) without relevant comorbidities were recruited from the general population. Glucocorticoid metabolites were measured by gas chromatography with tandem mass spectrometric detection in 24-hour urine collections to estimate total glucocorticoid production (TGP). Fasting plasma (apo)lipoproteins were assayed by routine methods.

Results: TGP was not decreased but tended to be increased in subjects with low HDL-C (NCEP-ATPIII criteria; $P = .094$). In univariate analysis, TPG was correlated inversely with HDL-C ($\beta = -0.353$, $P < 001$) and apoA-I ($\beta = -0.263$, $P = .01$). Multivariable linear regression analysis demonstrated that TGP was still inversely related to HDL-C ($\beta = -0.145$, $P = .019$) or alternatively to low HDL-C ($\beta = -0.129$, $P = .013$) taking age, sex, current smoking, and other metabolic syndrome components into account.

Conclusion: In this population-based study, urinary glucocorticoid metabolite excretion was inversely associated with HDL-C. We found no evidence for an attenuated adrenal function in men and women with low HDL-C.

Introduction

Steroid hormones synthesized by adrenal glands play a key role in multiple physiologic processes including glucose metabolism, (inflammatory) stress responses, and the maintenance of fluid and electrolyte balance (1). Cholesterol is the precursor of adrenal steroid hormones including glucocorticoids (2). Cholesterol required for adrenal steroidogenesis can be derived from *de novo* intracellular synthesis, from intracellular catabolism of stored cholesteryl esters, as well as via uptake of cholesterol transported by circulating lipoproteins (3,4). Cholesterol trafficked within plasma lipoproteins is considered to represent an important source of cholesterol that is used for steroidogenesis by the adrenal glands (5–7). Accordingly, studies in rodents have delineated that impaired adrenal steroidogenesis is a feature of scavenger receptor class B, type 1 deficiency, the receptor that plays a pivotal role in cellular uptake of high-density lipoprotein (HDL)-cholesteryl esters (8–10). Likewise, attenuated adrenal function has been documented in a few human subjects with heterozygous scavenger receptor class B, type 1 deficiency (11). Interestingly, decreased urinary 17-ketogenic steroid excretion, suggestive of compromised adrenal function, has been suggested in men with low HDL-cholesterol (HDL-C) both with and without heterozygous lecithin:cholesterol acyltransferase (LCAT) or ATP-binding cassette transporter 1 (ABCA1) deficiency (7). Remarkably, no relationship of partial HDL-C deficiency with diminished urinary glucocorticoid metabolite excretion has been observed in women (12). Moreover, even mild glucocorticoid excess is likely to be associated with a greater waist circumference and higher triglycerides,(13,14) which are well known to be associated with low HDL-C (15,16).

In view of the uncertainties with respect to the relationships between low HDL-C and glucocorticoid metabolism, it is relevant to better delineate the relationship of HDL-C with cortisol production in men and women subjects without rare genetic deficiencies affecting HDL metabolism. The aim of the present cross-sectional study was to delineate the relationship of urinary steroid metabolite excretion, measured using a novel gas chromatography- tandem mass spectrometry (GC-MS/MS)-based method, with HDL-C levels among healthy subjects recruited from the general population.

Subjects and methods

Two-hundred forty subjects were selected from the LifeLines Cohort Study, a large population-based cohort study in the northern part of The Netherlands (13). The study had been approved by the Medical Ethics Committee of the University

of Groningen, The Netherlands, and all participants provided written informed consent. From 6 age decades (20–79 years), 20 men and 20 women were selected, resulting in a cohort of 120 men and 120 women. The 60 women aged >50 years were considered to be postmenopausal. None of the subjects had reported comorbidities, and none used medications. Women using oral contraceptives were excluded. Biometric data were collected by a trained technician. Blood pressure was recorded every minute for 10 minutes using automated Dinamap Monitor (GE Healthcare, Freiburg, Germany) and the average of the last 3 readings was determined. Blood was drawn after overnight fasting between 8.00 and 10.00 AM. A 24-hour urine collection was obtained from all participants.

Laboratory methods

Total cholesterol and HDL-C levels were determined using an enzymatic colorimetric method, low-density lipoprotein-cholesterol (LDL-C) using a homogenous enzymatic colorimetric assay and triglycerides using an enzymatic colorimetric method, all on a Roche Modular P chemistry analyzer (Roche, Basel, Switzerland). For cholesterol, 1 µmol/L corresponds to 38.67 mg/dL; for triglycerides, 1 µmol/L corresponds to 88.5 mg/dL. Apolipoprotein (apo) A-I and B were determined using a nephelometric immunoassay (BN II, Siemens Healthcare Diagnostics, Germany). Glucose was measured by the hexokinase method; 1 mmol/L corresponds to 18 mg/dL.

Urinary steroid profiling using gas chromatography with tandem mass spectrometric detection (GC-MS/MS) was performed at the Laboratory, University Medical Centre Groningen of 24-hour urine collections of all subjects. Based on our elaborate experience with GC-MS,(17) we further improved this assay by developing a GC-MS/MS method, which measures 33 selected steroid metabolites to evaluate adrenal steroidogenesis (18). Cortisol is interconverted to cortisone by the 11β-hydroxysteroid dehydrogenase system, followed by reduction to tetrahydrocortisol (THF), allo-tetrahydrocortisol (allo-THF), and tetrahydrocortisone (THE), respectively. THF and allo-THF can then be further reduced to α- and β-cortols, whereas THE is reduced to cortolones (19). Total glucocorticoid production (TGP) was estimated as the sum of THF, allo-THF, cortols, and cortolones (20). Total androgen production (TAP) was estimated as the sum of A, E, DHEA, 11keto-E, 11OH-A, 11OH-E, 16OH-DHEA, 16keto-A2, 16keto-A3, and di- OH-DHEA.

Statistical analysis

Statistical analysis was done using SPSS (version 22; IBM Corporation, Armonk, NY, USA). Data are expressed as mean ± standard deviation or median with interquartile range as appropriate. To compare subjects with and without low HDL-C, cut-off

values according to NCEP-ATP III criteria were applied (<1.03 µmol/L for men <1.30 µmol/L for women) (21). Differences between groups were determined by unpaired *t*-test, Mann–Whitney U test, or Chi-square test where appropriate. Because of skewed distribution, logarithmically transformed values were used of TGP, TAP, and triglycerides for correlation analysis. Univariate relationships were determined using Pearson's correlation coefficients. Multivariable linear regression analyses were carried out to disclose the relationship of TGP with HDL-C or apoA-I taking account of age, sex, and smoking status, as well as systolic blood pressure, waist, glucose, and triglycerides, representing metabolic syndrome components. Two-sided *P* values >05 were considered significant.

Results

Clinical characteristics of the study population are shown in Table 1. Subjects were normotensive and their body mass index (BMI) was normal to slightly elevated. Fasting plasma glucose and average (apo)lipoprotein levels are listed in Table 1. LDL-C and triglycerides were slightly higher, whereas HDL-C and apoA-I levels were lower in men vs women. TGP and TAP were both higher in men (Table 1).

Table 1. Clinical characteristics, fasting glucose, (apo)lipoproteins, total glucocorticoid production (TGP), and total androgen production (TAP) in 120 men and 120 women recruited from the general population

Variable	Men (n= 120)	Women (n= 120)	P value
Age (y)	49.1 ± 16.8	49.5 ± 17.0	.843
Current smoking (yes/no)	26/94	24/96	.874
BMI (kg/m^2)	25.± 6 2.1	24.7 ± 2.4	.004
Waist (cm)	92.7 ± 7.4	84.6 ± 8.5	<0.001
SBP (mmHg)	131 ± 13	124 ± 16	<0.001
DBP (mmHg)	76 ± 8	72 ± 8	<0.001
Glucose (mmol/L)	5.1 ± 0.8	4.8 ± 0.5	.004
Total cholesterol (mmol/L)	5.14 ± 1.02	5.17 ± 1.13	.871
LDL-C (mmol/L)	3.44 ± 0.92	3.16 ± 0.95	.026
HDL-C (mmol/L)	1.37 ± 0.28	1.73 ± 0.46	<0.001
Triglycerides (mmol/L)	0.98 (0.70–1.28)	0.76 (0.58–1.06)	<0.001
ApoB (g/L) (*n*= 89)	0.93 ± 0.23	0.92 ± 0.23	.754
ApoA-I (g/L) (*n*= 89)	1.46 ± 0.23	1.70 ± 0.32	<0.001
TGP (mmol/24 h)	37.59 (31.61–44.75)	22.59 (18.94–29.93)	<0.001
TAP (mmol/24 h)	35.92 (25.43–47.62)	16.09 (12.04–25.79)	<0.001

lipoprotein-cholesterol; SBP, systolic blood pressure.

Data are expressed as mean ± standard deviation or median (interquartile range) with corresponding P value.

Conversion factors: for cholesterol 1 mmol/L corresponds to 38.67 mg/dL; for triglycerides 1 mmol/L corresponds to 88.5 mg/dL; for glucose 1 mmol/L corresponds to 18 mg/dL.

The percentage of subjects who met the criteria for low HDL-cholesterol was 11.6% (Table 2). Subjects with low HDL-C were younger, smoked more often, had a higher BMI and waist circumference, as well as lower total cholesterol levels. In men and women combined, TGP tended to be higher in subjects with low HDL-C compared with subject without low HDL-C (*P*= .094; Table 2; Fig. 1A). TGP was 40.97 (31.04–49.37) µmol/24 h and 37.59 (31.61–43.80) µmol/24 h in men with and without low HDL-C, respectively (*P*=.154; Fig. 1B). TGP was 25.97 (22.97–37.26) µmol/24 h in women with low HDL-C vs 22.16 (18.01–28.34) µmol/ 24 h in women without low HDL-C (*P*= .011; Fig. 1B). In men and women combined, TAP was higher in the subjects with low HDL-C vs subjects without low HDL-C (Table 2; Fig. 2A). TAP was 44.70 (30.05–53.15) µmol/24 h vs 35.00 (24.74–46.83) mmol/24 h in men with and without low HDL-C, respectively (*P*= .354; Fig. 2B). TAP was 27.51 (16.47–35.32) vs 15.96 (11.34–23.25) µmol/24 h in women with and without low HDL-C, respectively (*P*= .009; Fig. 2B).

Table 2. Clinical characteristics, fasting glucose, (apo)lipoproteins, total glucocorticoid production (TGP), and total androgen production (TAP) determined in a 24-hour urine collection in subjects with and without low HDL-cholesterol (HDL cholesterol <1.03 mmol/L for men and <1.30 mmol/L for women)

Variable	Low HDL cholesterol (*n*= 28)	No low HDL cholesterol (= 212)	*P* value
Sex (m/f)	12/6	108/104	.421
Current smoking (yes/no)	11/17	39/173	.015
Age (y)	41.0 ± 14.1	50.4 ± 6.9	.007
BMI (kg/m$^{2)}$	26.1 ± 2.3	25.0 ± 2.3	.020
Waist (cm)	89.7 ± 9.0	88.5 ± 9.0	.591
SBP (mmHg)	127 ± 15	128 ± 15	.965
DBP (mmHg)	73 ± 6	74 ± 8.5	.931
Glucose (mmol/L)	4.9 ± 0.5	5.0 ± 0.7	.926
Total cholesterol (mmol/L)	4.7 ± 1.1	5.2 ± 1.1	.016
LDL-C (mmol/L)	3.1 ± 1.0	3.3 ± 0.9	.237
HDL-C (mmol/L)	1.0 ± 0.1	1.6 ± 0.4	<.001
Triglycerides (mmol/L)	1.18 (0.85-1.69)	0.80 (0.61-1.15)	.002
ApoA-I (g/L) (*n*= 89)	1.22 ± 0.10	1.62 ± 0.29	<.001
ApoB (g/L) (*n* = 89)	0.95 ± 0.33	0.92 ± 0.22	.595
TGP (mmol/24 h)	31.89 (25.39-46.10)	30.28 (21.90-38.95)	.094
TAP (mmol/24 h)	32.37 (19.97-44.87)	24.31 (15.45-37.88)	.045

lipoprotein-cholesterol; SBP, systolic blood pressure.
Data are expressed as mean ± standard deviation or median (interquartile range) with corresponding P value.
Conversion factors: for cholesterol 1 mmol/L corresponds to 38.67 mg/dL; for triglycerides 1 mmol/L corresponds to 88.5 mg/dL; for glucose 1 mmol/L corresponds to 18 mg/dL.

In all subjects combined, TGP was correlated positively with BMI, waist, blood pressure, and glucose (Table 3). Positive correlations were also found of TGP with LDL-C, triglycerides, and apoB. Inverse correlations were found with HDL-C and apoA-I. In men, HDL-C was correlated negatively with TGP, whereas this relationship did not reach significance in women. In women, TGP was correlated positive with BMI, waist, blood pressure, glucose, and apoB. Current smoking was associated with higher TGP in all subjects combined (smokers: 34.03 [26.88–41.15] vs non-smokers: 28.72 [21.88–37.82] µmol/24 h, *P*= .011), as well as in women separately (28.94 [19.94–36.84] vs 22.26 [17.81–26.32] mmol/24 h, *P*= .011). Furthermore, TGP was positively related to TAP in all subjects combined, as well as in men and in women separately (Table 3).

Table 3. Univariate correlations of total glucocorticoid production (TGP) determined in a 24-hour urine collection with clinical characteristics and (apo)lipoproteins

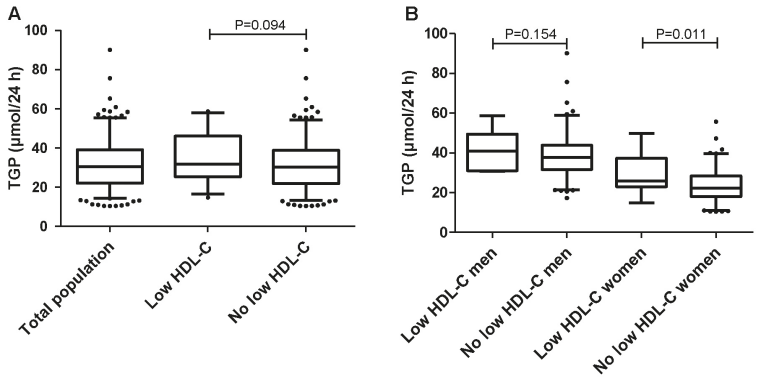
	Total population		**Men (*n*= 120)**		**Women (*n*= 120)**	
Variable	***R***	***P* value**	***R***	***P* value**	***R***	***P* value**
Age	0.077	.237	–0.043	.641	0.165	.072
BMI	0.312	<.001	0.118	.201	0.356	<.001
Waist	0.456	<.001	0.148	.108	0.328	<.001
SBP	0.298	<.001	0.134	.144	0.229	.012
DBP	0.239	<.001	0.034	.714	0.187	.041
Total cholesterol	0.066	.312	0.075	.414	0.102	.270
LDL-C	0.171	.008	0.106	.251	0.108	.239
HDL-C	–0.353	<.001	–0.210	.021	–0.092	.318
Triglycerides	0.188	.004	0.029	.757	0.073	.426
ApoB (*n*= 89)	0.266	.012	0.254	.105	0.331	.023
ApoA-I (*n*= 89)	–0.263	0.13	–0.046	.771	–0.052	.730
Glucose	0.270	<.001	0.118	.119	0.304	.001
TAP	0.594	<.001	0.465	<.001	0.313	.001

cholesterol; SBP, systolic blood pressure; TAP, total androgen production.
Data expressed as Pearson's correlation coefficients with corresponding P values. TGP, TAP, and triglycerides are logarithmically transformed.

Multivariable linear regression analysis was subsequently carried out to determine the independent relationship of TGP with HDL-C taking into account sex, age, smoking status, blood pressure, waist, glucose, and triglycerides (Table 4). TGP was independently and inversely related to HDL-C besides positive relationships of TGP with male sex, current smoking, waist, and glucose (Table 4, model 1). Similar inverse trends of TGP with HDL-C were observed in men and women separately

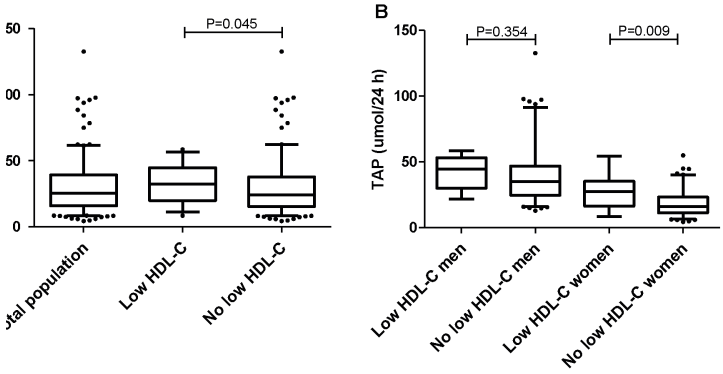
(Table 4, model 1). In an alternative model with apoA-I instead of HDL-C, TGP tended to be inversely associated with apoA-I (Table 4, model 2). Finally, in analysis with dichotomized HDL-C levels, TGP was also inversely related to low HDL-C, independent of associations with sex, waist, glucose, smoking status, and triglycerides (Table 4, model 3).

Figure 1: Total glucocorticoid production (TGP) determined in a 24-hour urine collection. (A) Subjects with or without low high-density lipoprotein cholesterol (HDL-C). (B) Men and women with or without low HDL-C.



Boxplots represent 25th and 75th percentiles with median, whiskers represent 5th and 95th percentiles, and dots represent outliers. P values are calculated for differences between groups.

Figure 2: Total androgen production (TAP) determined in a 24-hour urine collection. (A) Subjects with or without low high-density lipoprotein cholesterol (HDL-C). (B) Men and women with or with-out low HDL-C.



Boxplots represent 25th and 75th percentiles with median, whiskers represent 5th and 95th percentiles, and dots represent outliers. P values are calculated for differences between groups.

Table 4. Multivariable linear regression analyses in 120 men and 120 women with total glucocorticoid production (TGP) determined in a 24-hour urine collection as dependent variable

	Total population						**Men**				**Women**			
	Model 1 (*n*= 240)		**Model 2 (*n*= 89)**		**Model 3 (*n*= 240)**		**Model 1 (*n*= 120)**		**model 2 (*n*= 42)**		**Model 1 (*n*= 120)**		**Model 2 (*n*= 47)**	
Independent Variable	**β**	***P* value**	**β**	***P* value**	**β**	***P* value**	**β**	***P* value**	**β**	***P* value**	**β**	***P* value**	**β**	***P* value**
Age	0.007	.912	**0.218**	**.039**	-0.012	.842	-0.072	.455	-0.028	.893	0.091	.393	**0.454**	**.005**
Sex (men vs woman)	**-0.425**	**<.001**	**-0.428**	**<.001**	**-0.488**	**<.001**								
Smoking (yes/no)	**0.162**	**.001**	0.163	.070	**0.149**	**.003**	0.113	.224	0.013	.945	**0.267**	**.002**	0.250	0.66
SBP	0.095	.087	-0.015	.871	0.091	.103	0.099	.309	-0.161	.402	0.072	.462	-0.095	.514
Waist	**0.216**	**.001**	0.168	.104	**0.224**	**<.001**	0.152	.174	0.232	.286	**0.328**	**.001**	**0.293**	**0.35**
Glucose	**0.126**	**.021**	0.079	.415	0.122	**0.24**	0.143	.160	0.093	.624	**0.198**	**.035**	0.195	.174
Triglycerides	**0.131**	**.026**	0.015	.874	**0.155**	**0.41**	0.202	.073	0.215	.279	0.144	.130	0.127	.310
HDL-C	**-0.145**	**.019**					**-0.250**	**.021**			-0.142	.139		
ApoA-I			-0.080	.415					-0.050	.781			-0.798	.430
Low HDL-C					**-0.129**	**0.13**								

apoA-I, apolipoproteinAI; HDL-C, high-density lipoprotein-cholesterol; SBP, systolic blood pressure; TG, triglyceride. Statistically significant determinants of total glucocorticoid production are shown in bold print.

β: standardized beta. TGP and triglycerides are logarithmically transformed.

Model 1 includes age, sex, current smoking, TG, SBP, waist, glucose, HDL-cholesterol. Model 2 includes age, sex, current smoking, TG, SBP, waist, glucose, ApoA1.

Model 3 includes age, sex, current smoking, TG, SBP, waist, glucose, low HDL cholesterol (<1.03 mmol/L for men and <1.30 mmol/L for women).

Discussion

In this large, well-defined North European population, we delineated the relationship between HDL-C and TGP using state-of-the-art GC-MS/MS techniques to quantify urinary steroid metabolites. TGP tended to be increased in subjects with low HDL-C defined according to NCEP-ATPIII criteria. Furthermore, TGP was inversely correlated with HDL-C both in univariate and in multivariable linear regression analysis taking account of age, sex, smoking status, and metabolic syndrome components. In univariate analysis, TGP was also inversely related to apoA-I, the most abundant apolipoprotein component of HDL particles. The present study, therefore, essentially rules out that in adult subjects, lower plasma HDL-C levels contribute to decreased TGP under basal circumstances.

Ample evidence from animal studies emphasizes the importance of HDL for adrenal steroidogenesis (8-10). Recent observations suggest that genetically determined low HDL-C levels may coincide with attenuated corticoid metabolite excretion in men but not in women (7,12) These findings prompted us to determine the relationships of TGP with HDL-C in men and women recruited from the general population. For a proper comparison between the report by Bochem et al. in men(7) and the present study, it is essential to recognize that the HDL-C levels in the previous report averaged 0.8 mmol/L both in participants with and without heterozygous LCAT and ABCA1 mutations (7). In a subsequent article from the same group, HDL cholesterol averaged 0.9 mmol/L among women with LCAT and ABCA1 mutations (12). Thus, it is unlikely that the magnitude of the decrease in HDL-C in the subjects described in these reports (7,12) provides an important explanation for the discrepancy with the current findings that TGP was not lower in subjects with NCEP-ATPIII-defined low levels of HDL-C. Of potential importance, we examined only healthy individuals whereas Bochem et al. also included subjects with previous coronary heart disease, diabetes, or statin use (7,12). In addition, the observed discrepancy could at least in part be explained by differences in analytical methods to estimate corticosteroid production. In those previous reports, corticoid metabolite excretion was measured by gas chromatography, whereas we applied a more advanced technique, that is, gas chromatography with tandem mass spectrometry (7,12). Moreover, the decrease in steroidogenesis found in men with low HDL-C in the study by Bochem et al. was particularly based on an impaired urinary excretion of 17-ketogenic steroid metabolites, which predominantly reflects androgen metabolites, rather than on changes in 17-hydroxysteroids excretion, which predominantly reflect glucocorticoid metabolites (7,12,22,23). For

this reason, we also examined relationships between HDL-C and urinary androgen metabolite excretion. TAP, estimated as the sum of urinary androgen metabolites, was higher in subjects with low HDL-C. TAP was not different between men with or without low HDL-C in apparent discrepancy with those previous findings,(7) whereas in women, TAP was higher in the context of low HDL-C (12). We found a positive relationship between TGP and TAP in all subjects combined, as well as in men and women separately. Besides a contribution of the adrenal glands to androgen production along with glucocorticoid production, our observation could also reflect the recently described stimulatory effect of testosterone or its metabolites on ACTH secretion (24). Further studies are required to more precisely delineate the mechanisms responsible for this association.

The relationship of HDL-C with urinary glucocorticoid metabolite excretion has only been determined in a few population-based studies so far. In Scottish men and women, HDL-C was inversely associated with glucocorticoids independent from obesity (25). In a survey among Hispanic subjects, urinary glucocorticoid metabolite excretion was elevated in subjects with the metabolic syndrome and was also inversely correlated with HDL-C (26). In line with these findings, we found inverse relationships of TGP with HDL-C and apoA- I, besides positive relationships of TGP with obesity, blood pressure, glucose, LDL-C, and triglycerides. In multivariable linear regression analysis, the inverse relationship of TGP with HDL-C was independent of waist, triglycerides, and other metabolic syndrome components. Notably, the present study was aimed to determine the extent to which TGP was dependent on the HDL-C level. For this reason, the relationships of TGP (as dependent variable) with adiposity and plasma triglycerides (as independent statistical determinants) should be interpreted with caution. Nonetheless, we interpret the present data with regard to the inverse relationship of TGP with HDL-C to agree with the possibility that a high-normal glucocorticoid production could contribute to an unfavorable cardiometabolic risk profile via decreases in HDL-C, which is generally appreciated as a cardiovascular risk marker (27,28). Of further interest, we found a modest positive correlation of TGP with LDL-C. In comparison, a nonsignificant relationship of plasma total cholesterol with urinary glucocorticoid metabolite excretion was documented in the Scottish survey (25). Whether the association of TGP with LDL-C as found in the present study points to a contribution of circulating LDL to human steroidogenesis as suggested by attenuated adrenal function in subjects with familial hypobetalipoproteinemia is unknown at present (29). Also the relevance of the finding that LDL receptor deficiency in mice may result in impaired adrenal HDL uptake is unclear for the human situation (30).

Several other methodological aspects of our study need to be discussed. We consider it a strength that a large and well-defined group of men and women without relevant comorbidities was selected for our study with application of state of the art technique for urinary steroid profiling. In addition, several models with respect to the association of TGP with HDL-C showed consistent results. Clearly, the present findings do not necessarily hold true for other ethnicities given the design of the Lifelines cohort study with preferential inclusion of subjects of North European descent. Furthermore, it should be emphasized that the observational nature of our cross-sectional study does not allow us to address any cause–effect relationships.

In conclusion, in this population-based study, urinary glucocorticoid metabolite excretion was inversely associated with HDL-C. We found no evidence for an attenuated adrenal function in men and women with low HDL-C.

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Chapter 4

Cholesterol delivery to the adrenal glands estimated by adrenal venous sampling: an *in vivo* model to determine the contribution of circulating lipoproteins to steroidogenesis in humans

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Abstract

Background: Cholesterol, required for adrenal steroid hormone synthesis, is at least in part derived from circulating lipoproteins. The contribution of high-density lipoproteins (HDL) and low-density lipoproteins (LDL) to adrenal steroidogenesis in humans is unclear.

Objective: The aim of the study was to determine the extent to which HDL and LDL are taken up by the adrenal glands using samples obtained during adrenal venous sampling (AVS).

Methods: AVS was successfully performed in 23 patients with primary aldosteronism. Samples were drawn from both adrenal veins and inferior vena cava (IVC). HDL cholesterol (HDL-C) and lipoprotein particle profiles were determined by nuclear magnetic resonance spectroscopy. Apolipoprotein (apo) A-I and apoB were assayed by immunoturbidimetry.

Results: Plasma HDL-C and HDL and LDL particle concentrations (HDL-P and LDL-P) were not lower in samples obtained from the adrenal veins compared with the IVC (HDL-C, $P = .59$; HDL-P, $P = .06$; LDL-P, $P = .93$). ApoB was lower in adrenal venous plasma than in IVC ($P = .026$; $P < .05$ for right adrenal vein). In 13 patients with an aldosterone producing adenoma (APA), apoB was also lower ($P = .045$) and LDL-P tended to be lower ($P = .065$) in the APA adrenal vein compared with the IVC. ApoA-I was not lower in adrenal venous plasma compared with the IVC, neither in the whole group ($P = .20$) nor in the APA subgroup ($P = .075$).

Conclusion: These *in vivo* observations suggest that circulating LDL may contribute to adrenal steroidogenesis in humans as inferred from adrenal venous-IVC apoB concentration differences. AVS is a feasible method to investigate the relationships between lipoproteins and steroidogenesis.

Introduction

Steroidogenesis by the adrenal glands is a complex enzymatic process by which cholesterol is converted to biologically active steroid hormones (1). Cholesterol, targeted for the synthesis of adrenal glucocorticoids, mineralocorticoids, and androgens, originates from *de novo* intracellular synthesis, from intracellular catabolism of stored cholesteryl esters, as well as from uptake of cholesterol carried by circulating lipoproteins (1,2). It has been assumed that cholesterol derived from circulating lipoproteins represents an important source for adrenal steroidogenesis (3–6).

Studies in mice have indicated that scavenger receptor class B, type I (SRBI) plays a pivotal role in the selective uptake of cholesteryl esters from high-density lipoproteins (HDL) particles, which are subsequently stored intracellular and converted into free cholesterol (7,8). Accordingly, SRBI deficiency in rodents results in impaired adrenal steroidogenesis (9–11). Mildly impaired adrenal function has also been documented in human subjects with heterozygous SRBI deficiency (12). Moreover, it has been suggested that adrenal glucocorticoid production could be impaired in the context of low HDL cholesterol (HDL-C) due to heterozygous deficiency of lecithin–cholesterol acyltransferase (LCAT), which catalyzes the esterification of cholesterol, or to adenosine triphosphate–binding cassette transporter 1 (ABCA1) deficiency, a transmembrane receptor that facilitates cholesterol efflux from cells to (nascent) HDL particles (5). Apolipoprotein (apo) B-containing lipoproteins, in particular, low-density lipoproteins (LDL), are considered a potentially important source of cholesterol for adrenal steroidogenesis as well (13–15). These particles are taken up by LDL receptor–mediated endocytosis with subsequent degradation and intracellular release of cholesterol (1). Adrenal function was, however, found to be uncompromised in subjects with heterozygous LDL receptor deficiency,(16) although modestly impaired adrenal function has been documented in abetalipoproteinemia, which is characterized by the absence of plasma apoB-containing lipoproteins (17).

Little is known about the contribution of circulating lipoproteins to adrenal glucocorticoid synthesis in humans without genetic abnormalities affecting plasma levels or binding capacities of HDL and LDL. Adrenal venous sampling (AVS) is currently recommended as the preferred diagnostic procedure in patients with primary aldosteronism to differentiate between a unilateral aldosterone producing adenoma (APA) and bilateral adrenal hyperplasia (BAH) (18). This procedure provides a unique opportunity to compare HDL and LDL particle concentrations and

characteristics in plasma obtained from the adrenal veins with the infra-adrenal inferior vena cava (IVC). We tested whether such lipoprotein measurements could provide an *in vivo* estimate of lipoprotein cholesterol uptake by the adrenal glands.

Subjects and methods

The studies were performed in a university hospital setting and have been exempted for approval according to the Dutch Medical Research Involving Human Subjects Act. This report is based on patient data and material acquired during routine care. Study subjects were hypertensive patients with biochemically confirmed primary aldosteronism (ie, elevated plasma aldosterone-renin ratio and nonsuppressible 24-hour urinary aldosterone excretion after a 3-day salt loading test) who underwent a successful AVS procedure for subtype classification. AVS was performed according to international recommendations (19). In brief, tetracosactide (Synacthen) was administered intravenously during the procedure at an infusion rate of 50 mg/h starting 30 minutes before the procedure. The sequential blood sampling technique was performed, starting with catheterization of the right adrenal vein. Peripheral blood samples were drawn from the infra-adrenal part of the IVC. Catheter positioning was checked fluoroscopically using an iodine-containing X-ray contrast agent (Iomeron300) and confirmed with a rapid intraprocedural plasma cortisol measurement. A selectivity index (ie, plasma $\text{cortisol}_{\text{side}}$/ plasma $\text{cortisol}_{\text{IVC}}$) >3.0 confirmed that the blood sample was taken from the adrenal vein (18). Lateralization of aldosterone secretion was considered to be compatible with the presence of an APA when the lateralization index (ie, plasma $\text{aldosterone}_{\text{dominant}}$/plasma $\text{cortisol}_{\text{dominant}}$: plasma $\text{aldosterone}_{\text{nondominant}}$/ plasma $\text{cortisol}_{\text{nondominant}}$) was ≥4.0 and the contralateral suppression index (ie, plasma $\text{aldosterone}_{\text{nondominant}}$/plasma $\text{cortisol}_{\text{nondominant}}$: plasma $\text{aldosterone}_{\text{IVC}}$/ plasma $\text{cortisol}_{\text{IVC}}$) was <1.0 (18). The patients were studied after an overnight fast.

Laboratory methods

Plasma aldosterone was assayed with a competitive fixed-time solid-phase radioimmunoassay as described (Coat-a-Count; Siemens Medical Solutions Diagnostics (20). Cortisol was measured by electrochemiluminescence immunoassay (Roche Modular Systems, Mannheim, Germany). Nonfasting plasma levels of total cholesterol, HDL-C, and triglycerides obtained during outpatient clinic visits were measured by routine biochemical methods. Non-HDL cholesterol was calculated as the difference between total cholesterol and HDL cholesterol. Very low– density lipoprotein (VLDL), LDL, and HDL particle profiles were measured by

nuclear magnetic resonance (NMR) spectroscopy with the LipoProfile-3 algorithm, as described (LipoScience Inc; Laboratory Corporation of America Holdings Raleigh, NC) (21). Lipoprotein subclasses were quantified from the amplitudes of their spectroscopically distinct lipid methyl group NMR signals. Diameter range estimates were for VLDL (including chylomicrons if present): >60 nm to 27 nm, for LDL: 18 nm to 27 nm, and for HDL: 14 nm to 8.2 nm. The VLDL, LDL, and HDL particle concentrations (VLDL-P, LDL-P, and HDL-P) were calculated as the sum of the respective lipoprotein subclasses. Weighted-average VLDL, LDL, and HDL sizes were derived from the sum of the diameter of each subclass multiplied by its relative mass percentage based on the amplitude of its methyl NMR signal (21). Quantification of HDL-C was accomplished by converting NMR particle numbers to lipid mass concentration units, assuming that the lipoprotein particles have normal lipid content. HDL cholesterol values correlate well with chemically measured values (21).

ApoB and apoA-I were measured by immunoturbidimetric assay performed on an Olympus AU680 chemistry analyzer using reagents and standards from Beckman Coulter Inc. The intra-assay coefficients of variation of lipoprotein subfractions, HDL-C, and apolipoproteins were all ≤3%.

Methodological approach and statistical analysis

To determine the uptake of circulating lipoprotein particles by the adrenal glands, it is necessary that the (apo)lipoprotein concentrations in the adrenal arteries and adrenal veins are comparable. Because catheterization of the adrenal arteries is not possible, we used samples drawn from the IVC instead. We assumed that plasma (apo) lipoprotein concentrations in the IVC and the adrenal arteries are similar as is illustrated by equal (apo)lipopro- tein concentrations in the femoral artery and femoral vein (22). Thus, potential lipoprotein uptake by the adrenal gland was estimated by analyzing differences in plasma lipoprotein particle numbers and apolipoproteins in plasma samples between the adrenal veins and the IVC.

Data are expressed as median with interquartile range (IQR) or range. Differences between plasma (apo)lipopro- tein concentrations between each of the adrenal veins and the IVC were determined using Friedman's analysis of variance for paired observations with Duncan's correction for multiple measurements. In case of APA (unilateral aldosterone production as described previously), differences between the corresponding adrenal vein and the IVC were determined by Wilcoxon's tests. A 2-sided *P* value <.05 was considered significant.

Results

We included 12 men and 11 women between 2008 and 2015. Median age was 57 (IQR, 50–61) years and 24-hour urinary aldosterone excretion was 78 (IQR, 44–125) nmol/ 24 h (upper limit of normal: 37.6 nmol/24 h) after a 3-day salt loading test. Serum creatinine, electrolytes, plasma total cholesterol, non-HDL cholesterol, HDL cholesterol, and triglyceride levels obtained as part of routine medical care are shown in Table 1. Four patients used a statin at the time of AVS. The selectivity index was >3 in all catheterized adrenal veins (range: 3.12–50.66), indicating that the AVS procedure was technically successful in all cases. Based on the lateralization index and the contralateral suppression index, an APA was diagnosed in 13 subjects. The others were diagnosed with bilateral adrenal hyperplasia.

Table 1: Baseline characteristics of 23 subjects with primary aldosteronism

Sex (M/F)	11/12
Age (y)	57 (22-73)
BMI (kg/m^2)	29 (18-44)
Creatinine (mmol/L)	72 (55-204)
Sodium (mmol/L)	144 (137-148)
Potassium (mmol/L)	3.6 (2.3-4.5)
Serum aldosterone (pmol/L)	540 (220-3220)
PRC (ng/L)	2.5 (0.7-16.8)
Urinary aldosterone after SLT (nmol/24 h)	78 (37-522)
PA subtype (APA/BAH)	13/10
Total cholesterol (mmol/L)	4.70 (3.10-7.90)
Non-HDL cholesterol (mmol/L)	3.10 (1.50-6.50)
HDL cholesterol (mmol/L)	1.30 (0.80-3.00)
Triglycerides (mmol/L)	1.44 (0.53-5.16)

APA, aldosterone producing adenoma; BAH, bilateral adrenal hyperplasia; BMI, body mass index; HDL, high-density lipoproteins; PA, primary aldosteronism; PRC, plasma renin concentration, SLT, sodium loading test. Data are expressed as numbers or median (range).

In the whole group, no significant differences were found in the VLDL-P, LDL-P, HDL-P, or HDL-C concentrations between samples from the adrenal veins and the IVC (Table 2). In addition, VLDL, LDL, and HDL lipoprotein subfractions (data not shown) and sizes (Table 2) were not different between adrenal veins and IVC samples. Of note, the apoB concentration was lower in the right adrenal vein compared with the IVC. Plasma apoA-I concentrations were not different between samples from the adrenal veins and the IVC. Results were similar after excluding 4 statin using patients (data not shown).

Table 2: Lipoprotein particle concentrations, sizes, HDL-C, and apolipoproteins in the IVC and median differences in each adrenal vein compared with the IVC in 23 patients undergoing adrenal venous sampling

Apolipoprotein	IVC	Left adrenal vein, IVC	Right adrenal vein, IVC	*P* value*
VLDL-P (nmol/L)	44.0 (29.8 - 55.2)	1.7 (–5.5 - 4.6)	0.1 (–8.8 - 7.6)	.84
LDL-P (nmol/L)	878 (602 - 1100)	4 (–53 - 58)	9 (–89 - 74)	.93
HDL-P (nmol/L)	27.2 (24.1 - 29.6)	0.3 (–0.1 - 1.3)	0.9 (–0.3 - 1.9)	.06
VLDL size (nm)	49.7 (45.9 - 54.2)	– 0.4 (–2.6 - 1.7)	– 0.3 (–2.1 - 2.8)	.57
LDL size (nm)	21.1 (20.5 - 21.5)	– 0.3 (–0.6 - 0.1)	–0.15 (–4.23 - 0.2)	.13
HDL size (nm)	9.0 (8.5 - 9.5)	0.0 (–0.1 - 0.1)	0.0 (–0.1 - 0.1)	.93
HDL-C (mmol/L)	1.01 (0.85 - 1.32)	0.0 (0.0 - 0.5)	0.0 (–0.26 - 0.08)	.59
ApoA-I (g/L)	1.57 (1.40 - 1.74)	– 0.00 (–0.08 - 0.10)	– 0.03 (–0.09 - 0.05)†	.20
ApoB (g/L)	0.81 (0.64 - 0.94)	– 0.01 (–0.03 - 0.02)	– 0.02 (–0.04 - 0.01)†	.026

Apo, apolipoprotein; HDL-C, high-density lipoprotein cholesterol; HDL-P, high-density lipoprotein particle concentration; IVC, inferior vena cava; LDL-P, low-density lipoprotein particle concentration; VLDL-P, very low–density particle concentration. Data are expressed as median (interquartile range).

*P value: overall P value by Friedman's 2-way analysis of variance for paired observations with subsequent Duncan correction for multiple comparisons. †P < .05 from IVC.

We next tested whether lipoprotein uptake would be higher in adrenal glands harboring an APA. In 13 subjects diagnosed with APA, apoB was significantly lower in the adrenal vein at the side of the APA compared with the IVC (Table 3; Figure 1). Accordingly, LDL-P tended to be lower in the adrenal vein at the APA side compared with IVC. HDL-P, HDL-C, and apoA-I concentrations were not significantly different in the APA draining adrenal vein (Table 3).

Figure 1: Apolipoprotein B (apoB) concentrations in the inferior vena cava (IVC) and adrenal vein at the side were the aldosterone producing adenoma (APA) was located in 13 APA patients.

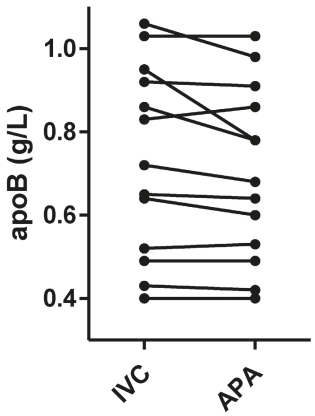

Table 3: Lipoprotein particle concentrations, sizes, HDL-C, and apolipoproteins in 13 patients diagnosed with an APA

Apolipoprotein	IVC	APA side, IVC	*P* value
VLDL-P (nmol/L)	35.9 (27.2 – 47.2)	3.0 (–8.8 – 6.7)	.650
LDL-P (nmol/L)	716 (574 – 1050)	–17 (–101 – 4.5)	.065
HDL-P (nmol/L)	26.8 (22.7 – 28.1)	0.3 (–0.2 – 1.2)	.077
VLDL size (nm)	48.5 (46.5 – 52.0)	–0.9 (–3.5 – 1.9)	.294
LDL size (nm)	21.2 (20.5 – 51.5)	–0.3 (–0.7 – 0.1)	.132
HDL size (nm)	9.1 (8.9 – 10.0)	0.0 (–0.1 – 0.1)	.348
HDL-C (mmol/L)	1.06 (0.87 – 1.31)	0.0 (0.0 – 0.1)	.672
ApoA-I (g/L)	1.65 (1.51 – 1.75)	–0.03 (–0.11 – 0.01)	.075
ApoB (g/L)	0.72 (0.51 – 0.94)	–0.01 (–0.06 – 0.00)	.045

APA, aldosterone producing adenoma; Apo, apolipoprotein; LDL-P, low-density lipoprotein particle concentration; HDL-C, high-density lipoprotein cholesterol; HDL-P, high-density lipoprotein particle concentration; VLDL-P, very low–density particle concentration.
Data are expressed as median (interquartile range). Median concentrations in the inferior vena cava (IVC) and median differences in the adrenal vein corresponding to the side of the APA are shown.

Discussion

Based on measurements of plasma (apo)lipoproteins and subfraction concentrations in samples from the adrenal veins and the IVC, we show here for the first time that the apoB concentration in adrenal veins is lower compared with the IVC. The apoB concentration was particularly lower in the adrenal vein at the side of the APA, conceivably as a result of the higher rate of steroid production by the adenoma. Accordingly, the decrease in the venous plasma LDL-P concentration at the APA side was close to statistical significance. Our findings, therefore, agree with the concept that steroidogenesis in humans may to some extent be driven by circulating LDL particles as supplier of cholesterol to the adrenal glands.

Neither HDL-C nor HDL-P and apoA-I were lower in adrenal venous plasma compared with the IVC. Moreover, HDL size was not decreased as would be expected if the adrenal glands were to a considerable extent using cholesteryl esters from circulating HDL via SRBI-mediated uptake (9,10,23). However, these findings should be interpreted with caution because we cannot entirely exclude that the lack of difference in HDL variables is attributable to a type II error. Thus, the extent to which circulating HDL may contribute to adrenal steroidogenesis as determined during tetracosactide-stimulated adrenal glucocorticoid output remains uncertain.

The AVS procedure was performed during stimulation with tetracosactide as recommended (19). This allowed us to delineate adrenal uptake of (apo)lipoproteins during maximally stimulated cortisol output. In comparison, SRBI mutation carriers showed mildly diminished cortisol stimulation in response to tetracosactide in particular when variation in cortisol binding globulin levels was taken into account,(12) whereas the cortisol response to tetracosactide was not attenuated in men with low HDL-C due to heterozygous lecithin–cholesterol acyltransferase or adenosine triphosphate–binding cassette transporter 1 deficiency (5). Of further relevance, no positive relationship between urinary cortisol metabolite secretion as an estimate of cortisol production, and plasma HDL-C has been documented in several population-based studies (24–26). Altogether, the importance of circulating HDL for adrenal steroidogenesis in subjects without rare genetic deficiencies affecting HDL metabolism is still uncertain.

It could be argued that our findings do not represent normal physiology as we examined subjects with primary aldosteronism under stimulating conditions with tetracosactide. However, it should be noted that, because of its invasive character and inherent risks, studies describing AVS in healthy subjects are not available. The continuous intravenous administration of tetracosactide stimulates the adrenal steroid biosynthesis, dampens the pulsatility of adrenal steroid secretion, and is, therefore, expected to enhance the uptake of circulating cholesterol, thereby improving the sensitivity to demonstrate relevant changes in the lipoprotein profile. Furthermore, the mechanism of cholesterol uptake by the adrenal gland is not likely to be different between patients with primary aldosteronism and healthy subjects.

Obviously, the interpretation of the present findings depends on the validity of the assumption that the plasma concentrations of LDL and HDL in the adrenal arteries and IVC are similar (22). It is likely that adrenal venous blood is to some extent mixed with accessory vein blood flow during the AVS procedure (19). This might diminish apparent adrenal venous-IVC differences in the plasma concentrations of the various lipoproteins, and hence result in underestimation of the magnitude of adrenal lipoprotein uptake. However, in the setting of highest expected difference (ie, at the APA side), we did find a significant gradient in apoB concentrations, which we regard as a proof of the concept that this novel approach is feasible to asses lipoprotein uptake *in vivo*.

In conclusion, the *in vivo* observations described here support the possibility that circulating LDL may contribute to adrenal steroidogenesis in humans.

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PART II

Adrenal medulla: optimization of current diagnostic strategies for PPGL

Chapter 5

Incidence of pheochromocytoma and sympathetic paraganglioma in the Netherlands: A nationwide study and systematic review

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Abstract

Introduction: Recent years have seen major changes in clinical practice which may have affected the incidence rates of pheochromocytoma(PCC)/sympathetic paraganglioma(sPGL). There is, however, a lack of up-to-date information describing trends in these incidence rates.

Methods: We searched the Dutch pathology registry to identify all histopathologically confirmed cases of PCC/ sPGL diagnosed between 1995 and 2015. We calculated incidence rates according to age category as well as age-standardized incidence rates (ASR). We also searched Medline and Embase to find data on nationwide incidence rates of PCC/sPGL.

Results: The nationwide pathology study revealed a total of 1493 patients with either PCC or sPGL. The ASR for PCC increased from 0.29 (95% CI: 0.24–0.33) to 0.46 (95% CI: 0.39–0.53) per 100,000 person-years in the periods 1995–1999 and 2011–2015, respectively. For sPGL the ASR in these same periods were 0.08 (95% CI: 0.06–0.10) and 0.11 (95% CI: 0.09–0.13) per 100,000 person-years, respectively. Concomitantly, PCC size decreased (β −0.17; $P < .001$) and age at diagnosis increased (β 0.13; $P = .001$). Our systematic search yielded 3 papers reporting on a total of 530 PCC/sPGL cases, showing a combined annual incidence rate varying from 0.04 to 0.21 per 100,000 person-years.

Conclusion: Incidence rates of PCC/sPGL have increased significantly over the past two decades. This trend coincides with a higher age and a smaller tumor size at diagnosis. Most likely these observations are at least in part the result of changes in clinical practice during the study period, with a more intensified use of both imaging studies and biochemical tests for detecting PCC/sPGL.

Introduction

Pheochromocytomas (PCC) and sympathetic paragangliomas (sPGL) are rare neuroendocrine tumors derived from chromaffin tissue of the adrenal medulla and the extra-adrenal sympathetic paraganglia, respectively. Histologically these tumors are identical and they share the capacity to synthesize and release catecholamines (dopamine, norepinephrine and epinephrine) (1–3). Uncontrolled hypersecretion of catecholamines by these tumors may evoke typical signs and symptoms such as paroxysmal hypertension, sweating and tachycardia, and can result in severe cardiovascular morbidity and mortality (4). Surgical resection is the treatment of choice, as it represents the only option for cure (5).

In the past, a substantial proportion of PCC/sPGL was not diagnosed during life but discovered post mortem during autopsy (6). Recent years have seen a tremendous rise in the number of imaging studies being ordered in clinical practice (7, 8), as well as more frequent assessment of metanephrines in plasma or urine (9). The sensitivity of biochemical testing and imaging techniques for detecting PCC/sPGL has also improved substantially over the past two decades (10–12). It is conceivable that these changes in diagnostic procedures have influenced the detection rate of PCC/sPGL during life in recent years. However, epidemiological data on PCC/sPGL, and particularly on its incidence rate, are surprisingly scarce.

Our objective was to determine the annual incidence rate of PCC/ sPGL during the past two decades in the Netherlands. To this end we conducted a retrospective nationwide pathology study. We hypothesized that the annual incidence rate has increased during the past two decades. For comparison of our data, we performed a systematic review of the literature on previously reported nationwide incidence rates of PCC/sPGL.

Methods

Systematic review

In order to identify articles published in peer-reviewed medical journals we conducted a systematic search of PubMed/MEDLINE and Embase, in agreement with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement (13). We used the following search terms: pheochromocytoma, paraganglioma, epidemiology, incidence, prevalence, autopsy, and post mortem examination (see Supplemental data for detailed information). The search was

carried out on November 10, 2016. We considered articles to be eligible for inclusion if they reported original research data on nationwide annual incidence rates of PCC and sPGL, or both, and were published in the English, German, French, Spanish or Dutch language. To avoid the risk of referral and migration bias we excluded papers reporting incidence rates derived from cases collected in one or more centers or only in a certain geographical region. We likewise excluded papers that exclusively described autopsy series without reporting estimates of nationwide incidence rates.

Two authors (A.B. and E.B.) independently and in duplicate assessed the eligibility of all papers. Titles and abstracts were screened first. Next, full-text articles were retrieved of potential relevant articles and these were thoroughly assessed. Articles were also searched for relevant references. If titles and abstract screening were inconclusive the full- text article was evaluated for eligibility. One reviewer extracted data including study design, national annual incidence rates of PCC/sPGL, and demographics of the study participants. The second reviewer checked the accuracy of the extracted data. The primary endpoint of this systematic review was the nationwide annual incidence rate of PCC/sPGL.

For each study we considered the following risks of bias: completeness and reliability of data acquisition and reporting of the primary endpoint, duration of the study period, and selection of the population. We graded the quality of the reported data according to the Oxford Centre for Evidence-Based Medicine levels of evidence (14).

Nationwide pathology study

We searched the Dutch Pathology Registry (PALGA) to identify all histologically proven PCC and sPGL diagnosed in the Netherlands between January 1, 1995 and December 31, 2015. The PALGA registry is a nationwide network and registry of histo- and cytopathology in the Netherlands, with coverage dating back to 1991 (15). We conducted a systematic analysis of the PALGA registry, using the following search terms: adrenal, adrenal medulla, pheochromocytoma(s) and paraganglioma(s). This search yielded a list of excerpts, i.e. summaries of the original pathology reports, including a limited amount of anonymized patient data. We labeled each excerpt with a unique patient identification number. It is worth noting that if the pathology material was obtained from different anatomical locations or at separate dates a single patient could have more than one excerpt in the PALGA registry. Two reviewers (A.B. and E.B.) independently evaluated all excerpts and included those describing a

diagnosis of PCC/sPGL. Excerpts reporting on pathology material offered for revision were excluded. We also excluded excerpts describing residual or recurrent disease, defined as a tumor resected from the same anatomical location as the first PCC/sPGL within or after 6 months of the initial resection, respectively. We also excluded metastatic lesions. A lesion was considered metastatic when PCC/sPGL tissue was reported to be present in nonchromaffin tissues such as lymph nodes, liver, lung or bones (3, 16). Furthermore, we excluded excerpts describing only cytology specimens and material offered for either additional immunohistochemistry staining or research purposes. Excerpts reporting a sPGL located in the aortopulmonary window were excluded as differentiation between sPGL and parasympathetic PGL was not feasible in the absence of clinical information (3). If the information provided in the excerpt was not sufficient, the original histopathology report was requested for further study. If the original histopathology report did not permit a definite diagnosis, an experienced pathologist (R.K.) re-examined the original pathology specimens.

Demographic data and tumor specifications were extracted from the excerpts sPGL were further subdivided by localization according to the World Health Organization (WHO) classification of endocrine tumors (3). A spinal localization was defined as a well-demarcated intradural or extradural mass without infiltration of spinal cord, soft tissues or bone (17). Bilateral PCC was subdivided into synchronous and metachronous presentation, defined as resection of the second PCC less or $>$ 6 months after the preceding contralateral adrenalectomy, respectively. In accordance with the Dutch Medical Research Involving Human Subjects Act, this study has been exempted from approval by the medical ethics committee.

For each study year we calculated age-specific incidence rates for PCC, sPGL, and PCC/sPGL combined. We subsequently determined an age-standardized incidence rate (ASR) for each year by calculating a weighted mean of the age-specific incidence rates, according to the standardized European population, in order to correct for changes in age distribution over time (18). In order to comprehensively delineate age-specific changes, we defined larger age categories as follows: 0–24, 25–49, 50–74, and 75 years or older. Age group specific incidence rates and their ratios were determined. We also calculated ASR and age- group specific incidence rates over the first and last 5 years of the study period. We obtained the required demographic data for these calculations from Statistics Netherlands.

In order to investigate a possible shift from post mortem towards ante mortem diagnosis, we calculated the incidence rate of post mortem diagnosed PCC/sPGL

per annum. Annual autopsy rates were calculated as the percentage of deceased individuals per annum in the Dutch population in whom an autopsy had been performed. We derived the number of clinical autopsies performed for each year from PALGA, in collaboration with the Dutch Society for Pathology.

Data are expressed as mean with 95% confidence interval (CI) or median with interquartile ranges [IQR], where appropriate. Distributions were analyzed using the Chi-square test. Univariate relationships were determined using Pearson's or Spearman's correlation coefficients, where appropriate. Multivariate linear regression analyses were carried out to disclose the relationship between year of diagnosis, post mortem incidence rate, and autopsy rate. Statistical analyses were performed using SPSS version 23.0 for Windows (IBM Corporation, Chicago, IL, USA). A two-sided P-value $< .05$ was considered significant.

Results

Systematic review

After removal of duplicates, the literature search yielded 2095 papers. After reading titles and abstracts we excluded 2025 articles. We excluded an additional 67 papers after reading the full texts (Supplemental Fig. 1). We finally included three papers in the present systematic review (19–21).

The three publications included in this systematic review were published between 1964 and 1988. Two of the three used a national disease registry for data extraction. One study used a questionnaire to identify cases retrospectively and probably suffered from recall bias. Nevertheless, we considered the risk of publication bias and selective reporting to be low.

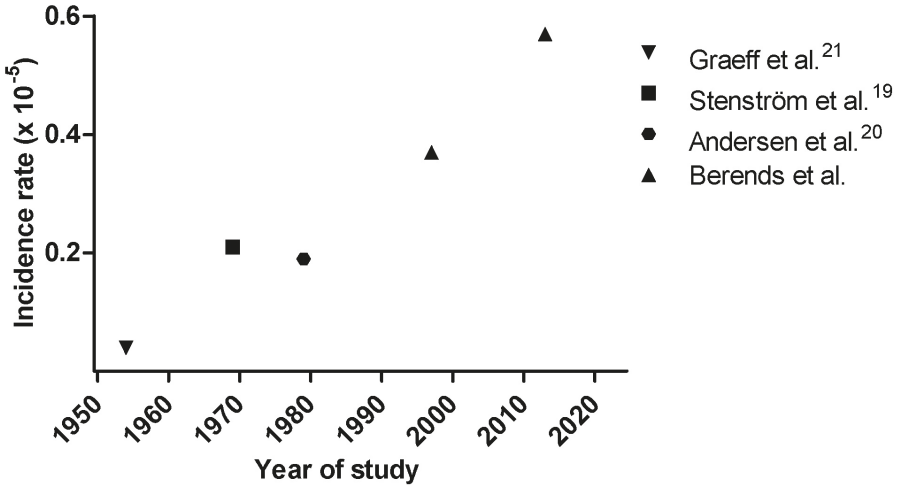
Collectively, these three studies comprised a total of 530 PCC/sPGL cases, with a mean observation period of 13 years. The reported crude incidence rates of PCC/sPGL in these studies varied between 0.04 per 100,000 and 0.21 per 100,000 person-years. Mean age at time of diagnosis varied between 43 $\pm$ 17 and 56 $\pm$ 18 years. The majority of the described tumors were PCC (80–100%). Reported localizations of sPGL were intra-abdominal/retroperitoneal (40–64%), intra-thoracic (3.5–60%) and in the urinary bladder (3.5%). In 17–42% of cases, the PCC/sPGL had remained undetected until autopsy (Table 1). The annual incidence rate of PCC/sPGL reported in these three papers and in the present study is depicted in Fig. 1.

Table 1: Overview of previous nationwide patient series with PCC/sPGL.

Study description					Population studied		Study outcomes					
Author/ year	**Country**	**Study design**	**Data collection**	**Data source**	**M:F**	**Age (yrs)**	**Total number PCC/ sPGL**	**Mean annual incidence ($\times 10^{-5}$)**	**Diagnosis post mortem %**	**Localization (%)**		**Level**
Stenström, 1986	Sweden	Retrospective (multicenter)	1958–1981	National disease registry	1:1.4	56 (± 18)[a]	439	0.21	42	Adrenal	78.4	2b
										Extra-adrenal	21.6	
										Intra-abdominal	64	
										Intra-thoracic	3.5	
										Urinary bladder	3.5	
										Miscellaneous	29	
Andersen 1988	Denmark	Retrospective (multicenter)	1977–1981	National disease registry	1.4:1	M: 45 [15–73][b] F: 50 [15–81][b]	47	0.19	17	Adrenal	100	2b
Graeff, 1964	The Netherlands	Retrospective (multicenter)	1949–1959	National questionnaire	1:1.2	43 (± 17)[a]	44	0.04	36	Adrenal	79.5	4
										Extra-adrenal	11.4	
										Intra-abdominal	40	
										Intra-thoracic	60	
										Miscellaneous	9.1	

Abbreviations: M, male; F, female; yrs., years; PCC, pheochromocytoma; sPGL, sympathetic paraganglioma. Age is described as mean (s± SD)[a] or median [range][b]. Mean annual incidence rates are presented per 100,000 person-years.

Figure 1: Nationwide incidence rates from literature and present study. Incidence rate presented per 100,000 person-years.



Year of study presented as median year of data collection period for the three earlier reports. For the present study, incidence rates are shown at start and end of data collection period (1995 and 2015, respectively).

Nationwide pathology study

The search through the PALGA registry yielded a total of 2871 excerpts. After processing, we included 1899 excerpts, corresponding to 1493 unique patients (Supplemental Fig. 2). The distribution of pathology diagnoses was as follows: PCC (n = 1210), sPGL (n = 274), synchronous presentation of PCC and sPGL (n = 9). Mean age at presentation was 51 ± 16 years with a wide range varying from 0 to 88 years (Fig. 2).

PCC/sPGL occurred more frequently in females than in males (55% vs.45%, P < .001). Among the 1210 patients with PCC, 1114 (92%) had a unilateral localization originating in the right (53%) or left (47%) adrenal gland (P = .026). The median diameter of PCC was 4.0 [2.5–6.5] cm. Thirty-eight patients (3.1%) with a PCC developed an additional PCC in the contralateral adrenal gland at least 6 months after the primary diagnosis (Table 2). Localizations of sPGL were intra-abdominal/retroperitoneal (56%), spinal (22%), in the urinary bladder (10%), intra-thoracic (9)% or miscellaneous (3%) (Table 2). The miscellaneous group consisted of sPGL originating in the mesentery, uterus, ovary, testicle or spermatic cord.

Figure: 2: Age at diagnosis pheochromocytoma (PCC).

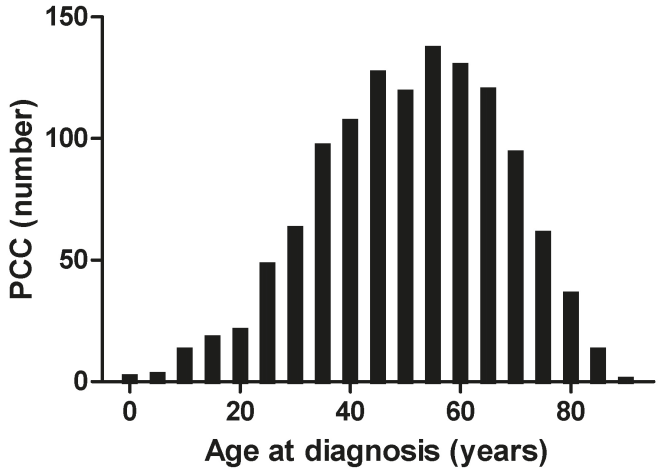
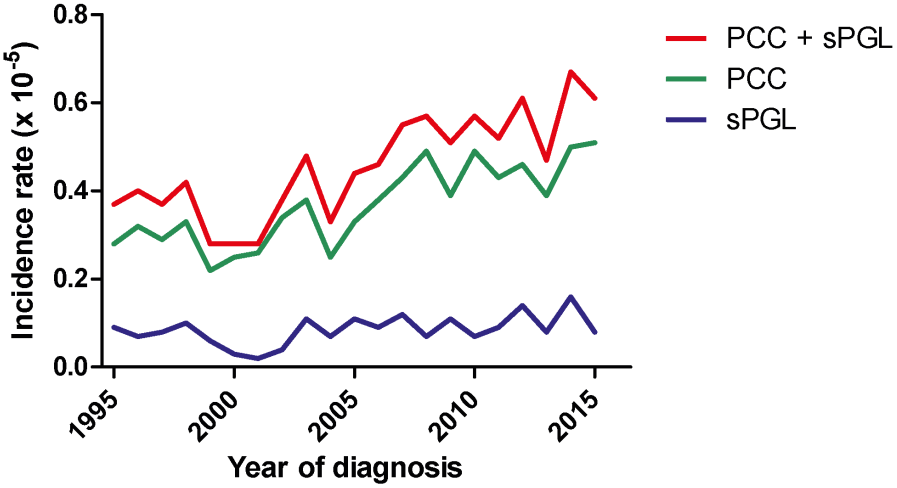


Figure 3: Annual age-standardized incidence rates (ASR) per 100,000 person-years of pheochromocytoma (PCC)/sympathetic paraganglioma (sPGL).



Combined PCC + sPGL (β 0.80, P < .001), PCC (β 0.82, P < .001) and sPGL (β 0.45, P = .04) in the Netherlands during 1995–2015.

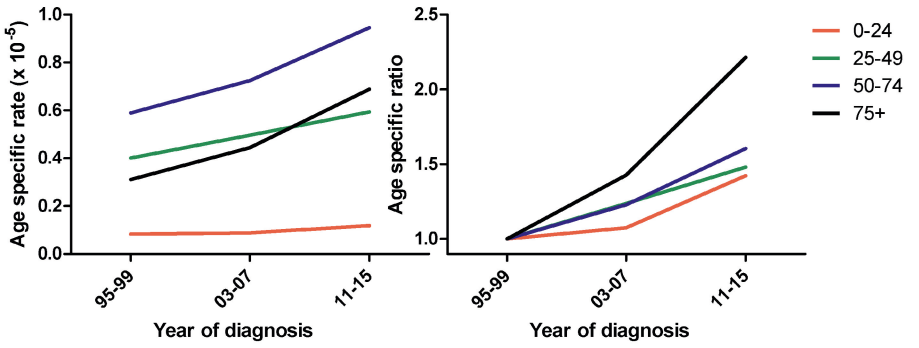
The overall ASRs of PCC/sPGL were 0.37 (95% CI: 0.31–0.43) and 0.57 (95% CI: 0.49–0.66) per 100,000 person-years in the period 1995–1999 and 2011–2015, respectively. The annual ASR of PCC/sPGL increased significantly during the study period (β 0.80, P < .001).

The overall ASRs of PCC were 0.29 (95% CI: 0.24–0.33) and 0.46 (95% CI: 0.39–0.53) per 100,000 person-years in the periods 1995–1999 and 2011–2015, respectively. For sPGL the overall ASRs were 0.08 (95% CI: 0.06–0.10) and 0.11 (95% CI: 0.09–0.13) per 100,000 person-years, respectively. The annual ASR of PCC increased during the whole study period (β 0.82, P < .001), whereas the annual ASR of sPGL increased (β 0.45, P = .04) to a lesser extent (Fig. 3). We observed the largest relative increase in age-specific incidence rates of PCC/sPGL in patients ≥75 years with a ratio of 2.2 in the last 5 years, compared to the first 5 years of the study period. During the entire observation period, the incidence rate was greatest among subjects in the age group 50–74 years (Fig. 4).

Age at diagnosis increased, (β 0.12; P < .001) while PCC tumor size decreased significantly (β −0.17; P < .001) during the study period (Figs. 5 and 6, respectively). In a multivariable linear regression model with study year as dependent variable, these correlations remained significant (PCC size: β −0.17; P < .001, age: β 0.13; P = .001).

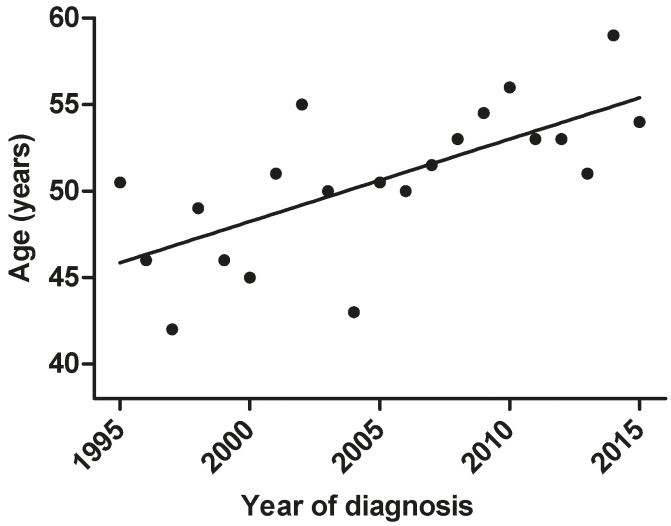
A total of 72 (4.8%) PCC/sPGL were diagnosed during post mortem examination. The mean annual incidence rate of post mortem diagnosed PCC/sPGL was 0.02 (95% CI: 0.016–0.027) per 100,000 person- years. Information on the number of autopsies performed in the Netherlands was available for the years 1995 through 2013. There was a strong decline in autopsy rate during this period (β −0.99; P < .001). Post mortem incidence of PCC/sPGL did not change during the study period (β −0.02; P = .612) after correction for the decrease in annual autopsy rate.

Figure 4: Absolute increase of age specific incidence rate (left) and relative increase of age specific incidence rate (right) stratified according to age group during the first, middle, and last 5 years of study period



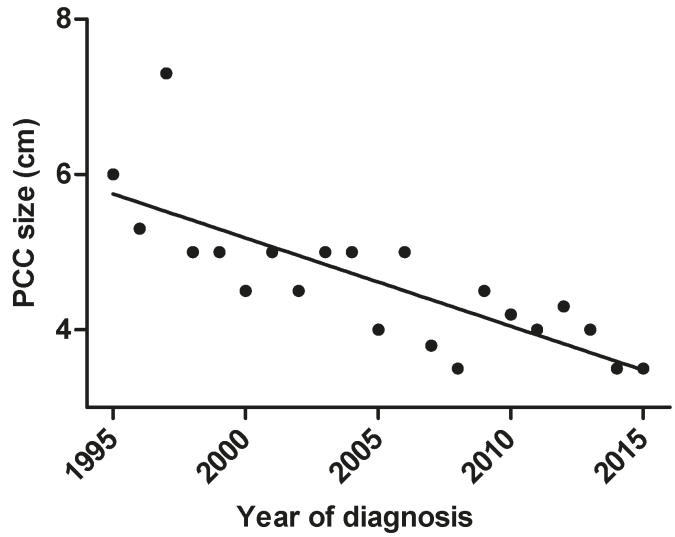
Incidence rate per 100,000 person-years.

Figure 5: Median age at diagnosis of pheochromocytoma.



Significant increase of age during whole study period (β 0.12; P < .001).

Figure 6: Median size of pheochromocytoma (PCC) at diagnosis.



Significant decrease of PCC tumor size during whole study period (β –0.17; P < .001).

Table 2: Characteristics of the present cohort.

	PCC (*n* = 1210)		**sPGL (*n* = 274)**		**Synchronous PCC and sPGL (n = 9)**	
Gender (M:F %)	43:57		53:47		44:56	
Age (yrs)	51 ± 16		51 ± 16		37 ± 16	
Localization	Right	43%	Abdominal/retroperitoneal	56%	Adrenal/abdominal	66%
	Left	49%	Spinal	22%	Adrenal/thoracic	22%
	Bilateral synchronous	5%	Urinary bladder	10%	Adrenal/abdominal/thoracic	11%
	Bilateral metachronous	3%	Thoracic	9%	Bilateral adrenal/abdominal	11%
			Miscellaneous	3%		

Abbreviations: M, male; F, female; yrs., years; PCC, pheochromocytoma; sPGL, sympathetic paraganglioma.

Discussion

We here describe the epidemiology of PCC/sPGL in the Netherlands during two decades, i.e. from 1995 to 2015. We found a significant increase of the ASR of PCC/sPGL, which coincided with a reduction in tumor size and a higher age at the time of initial diagnosis.

The incidence rates in the present study are derived from the largest series of patients with PCC/sPGL published so far, and are considerably higher than previously reported. There is, however, a lack of good quality estimates with respect to the epidemiology of PCC/sPGL. Despite a comprehensive review of the literature, we were able to retrieve only three papers reporting on nationwide incidence rates of PCC/sPGL (19–21). These previous studies were much smaller in size and difficult to compare with the present study because of differences in study design and population composition. Moreover, the reported incidence rates were not standardized for age distribution, thereby precluding reliable comparisons with contemporary populations. Nevertheless, the collective data from those earlier publications and the present study clearly suggest an increase in the annual incidence rate of PCC/sPGL over the past 60 years, as shown in Fig. 1.

Theoretically, the increased incidence rate of PCC/sPGL could reflect a true increase in the number of patients affected by this disease, a change in diagnostic practices resulting in earlier detection, or a combination of these. The observed increase in the incidence rate diagnosed during life combined with an unaffected post mortem incidence rate might support the contention that the number of patients developing a PCC/sPGL has actually increased.

Changes in tumorigenesis could be responsible for such an increased occurrence. For example, the rising incidence of papillary thyroid carcinoma has been linked to an increase in the frequency of somatic mutations in BRAF and RAS proto-oncogenes (22, 23). Recent genetic analysis in PCC/sPGL also identified somatic BRAF and RAS mutations as pathogenic, but it is currently unknown whether the occurrence of these mutations in PCC/sPGL has changed over the course of time (24). It can be questioned, however, whether the post mortem incidence rate of PCC/sPGL has indeed been stable over the years, as the number of autopsies fell dramatically in the Netherlands during the study period. A similar trend has also been reported in several other countries (25–28). This means that the selection of deceased individuals who are ultimately subjected to an autopsy procedure has

changed considerably over time, which will inevitably confound any attempt to extrapolate results from post mortem incidence rates to the general population (28, 29).

Diagnostic practices have changed profoundly in recent years and it seems plausible that these changes have largely contributed to the observed increase in the incidence rates of PCC/sPGL. The concomitant decrease in tumor size is also in support of a shift to an earlier diagnosis of PCC/sPGL. These trends correspond with recent studies describing increasing incidence rates of neuroendocrine tumors, in particular of localized disease, most likely as a result of earlier detection (30–32). The exponential growth in the use of various imaging studies (e.g. ultrasound, computed tomography, magnetic resonance imaging, positron emission tomography scanning) obviously increases the chance of visualizing an unexpected lesion such as an adrenal gland tumor (7,8,33). It has been demonstrated that a substantial number of these so called adrenal incidentalomas harbor a PCC (34–36). We found that both the absolute incidence rates and the relative increase in incidence rates were greatest among subjects older than 50 years, which is in agreement with the fact that most imaging studies are performed in this age group (7). Diagnostic practices during the study period were also altered by the introduction of a routine laboratory assay to determine metanephrines in either plasma or urine (9). This assay has significantly improved the accuracy of the biochemical diagnosis of PCC/ sPGL and has become the recommended first line test (5). In addition, DNA mutation screening programs have become standard practice as a direct consequence of the expanding number of identified mutations in PCC/sPGL susceptibility genes. Currently, about 30–40% of all PCC/ sPGL are considered to be hereditary, and annual biochemical screening with measurement of metanephrines in germline mutation carriers is now considered standard clinical practice (5,37,38).

It could be argued that earlier detection of PCC/sPGL would only lead to the diagnosis of indolent and therefore clinically irrelevant neuroendocrine tumors. However, there are several lines of evidence to support the clinical importance of a timely diagnosis of PCC/sPGL. The study by Sutton et al. reported that cardiovascular disease was the main cause of death in patients in whom PCC had been an unsuspected autopsy finding. Notably, 27% of these patients had died of hypertensive or hypotensive crises precipitated by surgery for unrelated conditions (6). In addition, Stolk et al. demonstrated a 14-fold higher rate of cardiovascular events among patients with a PCC compared to patients with

essential hypertension (4). This elevated risk was most likely explained by exposure to the toxic effects of catecholamines, as no differences were found in blood pressure or other cardiovascular risk factors. Moreover, it has been shown that normotensive patients and hypertensive patients with a PCC had similar degrees of hemodynamic instability during adrenalectomy (39). Collectively, these data underscore the importance of timely diagnosis and treatment of PCC.

Our study has some limitations. Unfortunately, we did not have access to clinical information on the study subjects and were therefore unable to analyze directly the potential determinants of the observed changes in incidence rates or to describe any relationships with morbidity, mortality or survival. Multifocal sPGL might be underreported in our study, since histopathology has probably not always been obtained from all tumor localizations. Another limitation was that the distinction between PCC and sPGL was not always clearly defined before the consensus report issued in 2004, which sometimes posed difficulties in interpretation of the terminology applied in older publications on incidence rates (3).

The strength of our study is the fact that the present results are derived from a nationwide pathology register, which precludes the potential selection bias that might occur in case of reports from one or a few referral centers. In addition, we provide novel epidemiological data on the incidence rates of PCC/sPGL over the past 20 years in the largest series of PCC/sPGL published until today.

In conclusion, the ASR of PCC/sPGL has increased significantly during the past two decades, most likely as a result of changes in diagnostic practices leading to earlier detection of these tumors. A higher detection rate of PCC/sPGL is of potential clinical importance, as earlier treatment is expected to reduce the elevated cardiovascular risk in these patients.

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Supplemental data

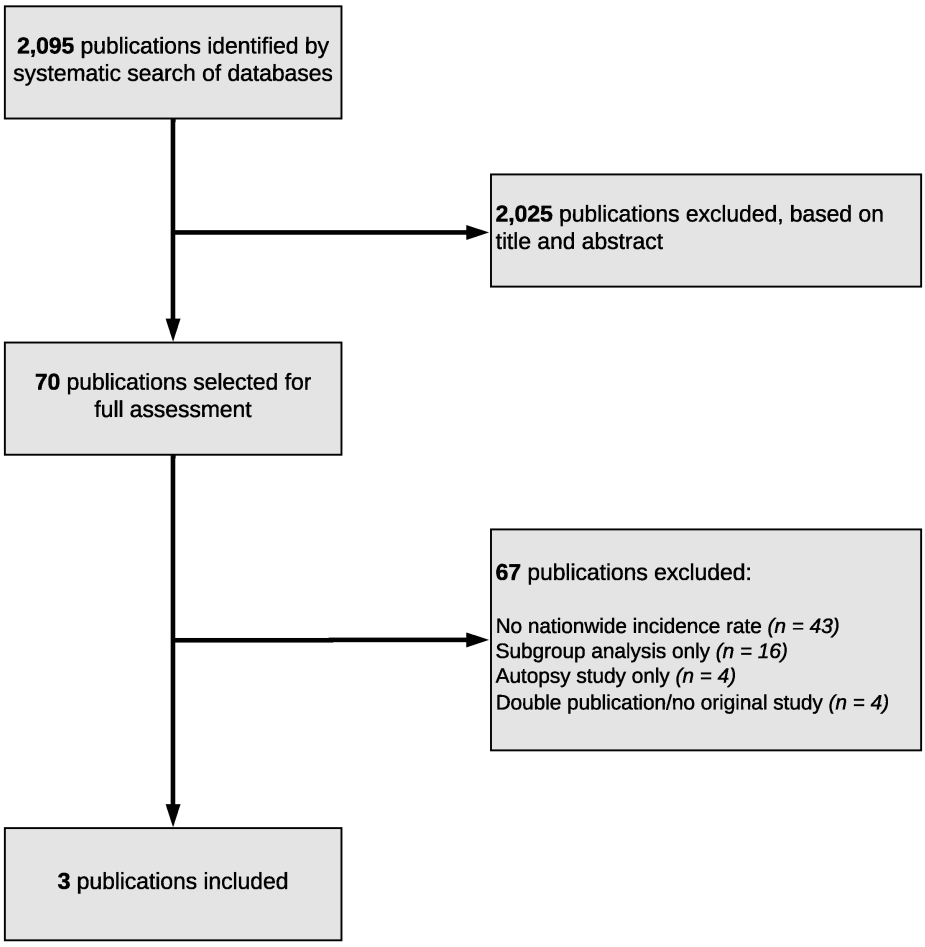
Supplementary information 1. Search strategy, full search string
Full search string by database.
Date search: November 10, 2016.

Search PubMed/Medline:
(((“Pheochromocytoma/epidemiology”[Mesh] OR Pheochromocytoma*[tiab] OR phaeochromocytoma*[tiab] OR phaechromocytoma*[tiab] OR “Paraganglioma/epidemiology”[Mesh:noexp] OR paraganglioma*[tiab]) AND (“Incidence”[Mesh] OR incidence[tiab] OR “Prevalence”[Mesh] OR prevalence[tiab] OR “Autopsy”[Mesh] OR autops*[tiab] OR Postmortem examination*[tiab])) NOT (“animals”[MeSH] NOT “humans”[MeSH])) NOT (((“Pheochromocytoma/epidemiology”[Mesh] OR Pheochromocytoma*[tiab] OR phaechromocytoma*[tiab] OR “Paraganglioma/epidemiology”[Mesh:noexp] OR paraganglioma*[tiab]) AND (“Incidence”[Mesh] OR incidence[tiab] OR “Prevalence”[Mesh] OR prevalence[tiab] OR “Autopsy”[Mesh] OR autops*[tiab] OR Postmortem examination*[tiab])) NOT (“animals”[MeSH] NOT “humans”[MeSH]))

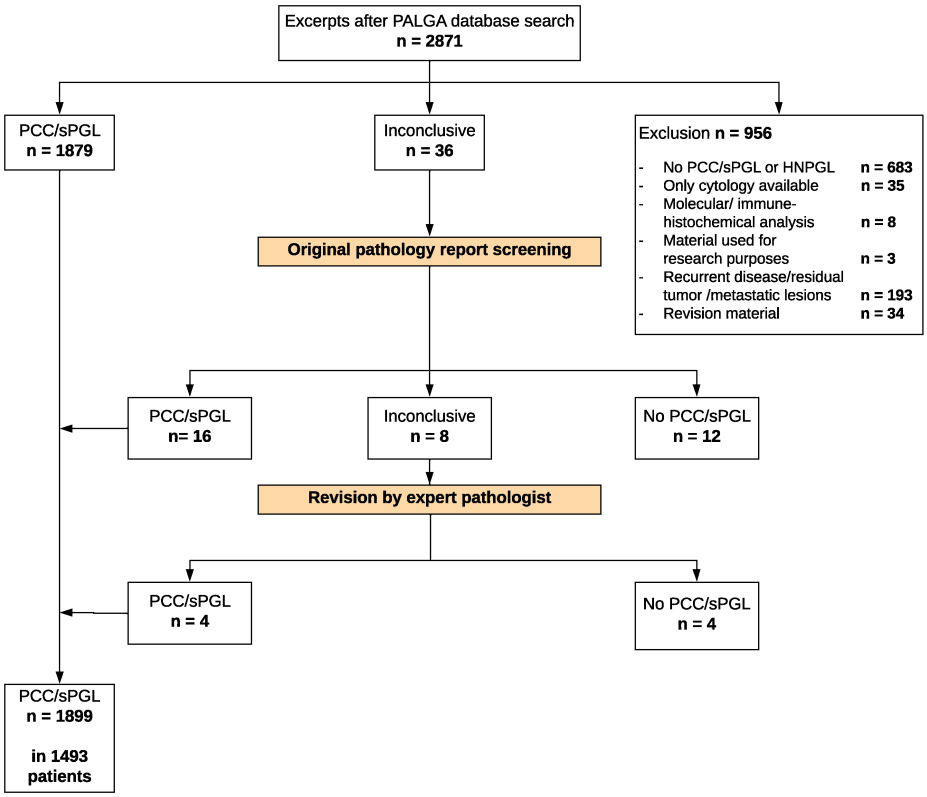
Search Embase:
(‘pheochromocytoma’/exp OR pheochromocytoma*:ab,ti OR phaeochromocytoma*:ab,ti OR phaechromocytoma*:ab,ti OR paraganglioma*:ab,ti AND (‘incidence’/exp OR ‘prevalence’/exp OR ‘autopsy’/exp OR incidence:ab,ti OR prevalence:ab,ti OR autops*:ab,ti OR ‘postmortem examination’:ab,ti) NOT (‘animal’/exp NOT ‘human’/exp)) NOT (‘pheochromocytoma’/exp OR pheochromocytoma*:ab,ti OR phaechromocytoma*:ab,ti OR paraganglioma*:ab,ti AND (‘incidence’/exp OR ‘prevalence’/exp OR ‘autopsy’/exp OR incidence:ab,ti OR prevalence:ab,ti OR autops*:ab,ti OR ‘postmortem examination’:ab,ti) NOT (‘animal’/exp NOT ‘human’/exp))

Supplemental figures

Supplemental Figure 1: Flow chart of search results and study selection.



Supplemental Figure 2: Flowchart of excerpt selection and processing.



Chapter 6

Unenhanced CT imaging is highly sensitive to exclude pheochromocytoma: A multicenter study

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Abstract

Background: A substantial proportion of all pheochromocytomas is currently detected during the evaluation of an adrenal incidentaloma. Recently, it has been suggested that biochemical testing to rule out pheochromocytoma is unnecessary in case of an adrenal incidentaloma with an unenhanced attenuation value ≤10 Hounsfield Units (HU) at computed tomography (CT).

Objectives: We aimed to determine the sensitivity of the 10 HU threshold value to exclude a pheochromocytoma.

Methods: Retrospective multicenter study with systematic reassessment of preoperative unenhanced CT scans performed in patients in whom a histopathologically proven pheochromocytoma had been diagnosed. Unenhanced attenuation values were determined independently by two experienced radiologists. Sensitivity of the 10 HU threshold was calculated, and interobserver consistency was assessed using the intraclass correlation coefficient (ICC).

Results: 214 patients were identified harboring a total number of 222 pheochromocytomas. Maximum tumor diameter was 51 (39–74) mm. The mean attenuation value within the region of interest was 36 ± 10 HU. Only one pheochromocytoma demonstrated an attenuation value ≤10 HU, resulting in asensitivity of 99.6% (95% CI: 97.5–99.9). ICC was 0.81 (95% CI: 0.75–0.86) with a standard error of measurement of 7.3 HU between observers.

Conclusion: The likelihood of a pheochromocytoma with an unenhanced attenuation value ≤10 HU on CT is very low. The interobserver consistency in attenuation measurement is excellent. Our study supports the recommendation that in patients with an adrenal incidentaloma biochemical testing for ruling out pheochromocytoma is only indicated in adrenal tumors with an unenhanced attenuation value >10 HU.

Introduction

Pheochromocytomas are rare neuroendocrine tumors arising from chromaffin tissue in the adrenal medulla (1). Timely diagnosis is crucial because patients are at increased risk for serious cardiovascular complications due to uncontrolled catecholamine hypersecretion (2, 3). In general, the presence of a pheochromocytoma is suspected in case of typical signs and symptoms such as paroxysmal headaches, palpitations, sweating and hypertension (4). The diagnosis of a pheochromocytoma is biochemically established by demonstration of elevated plasma or urinary levels of metanephrines, the *O*-methylated metabolites of catecholamines. However, a substantial proportion of patients with pheochromocytoma is either asymptomatic or may present with less typical signs and symptoms (5, 6). In such cases, a pheochromocytoma may be detected following the hormonal evaluation of an adrenal incidentaloma, which is defined as an adrenal mass >1 cm discovered incidentally during imaging procedures performed for reasons unrelated to adrenal disease (7). The reported prevalence of pheochromocytoma in adrenal incidentaloma cohorts is approximately 5–7% (8, 9). Conversely, nearly 30% of all pheochromocytomas are currently being diagnosed through the evaluation of adrenal incidentalomas (10). In view of these prevalence data, and the premise that a diagnosis of pheochromocytoma should not be missed, determination of metanephrines is recommended by the majority of guidelines on the management of adrenal incidentalomas (7, 9).

It has been suggested, however, that the likelihood of a pheochromocytoma is extremely low when the adrenal tumor demonstrates an unenhanced CT attenuation value ≤10 Hounsfield Units (HU). If this assumption would indeed be true, then expensive measurement of metanephrines could be reserved for adrenal incidentalomas with an unenhanced attenuation value above this threshold (11, 12, 13). In agreement with these observations, the recent guideline issued by the European Society of Endocrinology/European Network for the Study of Adrenal Tumors (ESE/ENSAT) states that it seems reasonable to avoid measuring metanephrines in case of an adrenal incidentaloma with an unenhanced attenuation value ≤10 HU (9). The quality of evidence behind this recommendation is very low, as only a few small-sized studies are available on this subject. Moreover, there are several reports describing pheochromocytomas with an unenhanced attenuation value ≤10 HU, suggesting that the sensitivity of this cut-off point might be suboptimal for application in clinical practice (14, 15).

We therefore aimed to systematically evaluate the characteristics of pheo-

chromocytoma at unenhanced CT scan in a large-sized study in order to determine the diagnostic value of this cut-off value to exclude the presence of a pheochromocytoma.

Subjects and methods

Patients with histologically proven pheochromocytoma diagnosed between the year 2000 and 2017 were identified in six university medical centers and one non-university teaching hospital in The Netherlands. Patients were considered eligible if the original preoperative unenhanced CT scan images of the adrenal glands were available for analysis. Recurrent or residual tumors were excluded. This retrospective study has been exempted from review and approval by the medical research and ethics committee of the University of Groningen, The Netherlands, according to the Dutch Medical Research Involving Human Subjects Act.

Clinical and biochemical data were extracted from the medical records and were anonymized before further analysis. Selected CT scans were reviewed independently by two experienced radiologists (T K and C H) using a standardized protocol. The radiologists were not blinded for the diagnosis of pheochromocytoma. Scanning acquisition and reconstruction parameters were extracted and the image quality was graded sufficient or insufficient for further analysis. The presence of movement or beam artifacts that interfered with attenuation measurement, incomplete visualization of the tumor and slice thickness >5 mm were considered as factors rendering the image quality insufficient. A two-dimensional region of interest (ROI) was drawn in the transversal plane by each radiologist avoiding the edges of the tumor and areas containing necrosis, calcifications and cystic formations. The radiologists had also access to the post-contrast series in case these were performed, which enabled more precise drawing of the ROI. The mean attenuation value, standard deviation of the attenuation value and ROI size were determined. In order to limit interobserver variability, a volume of interest (VOI) encapsulating the whole tumor was generated using a workstation (Syngo.via (version VB10B_HF06) Siemens Healthineers, Erlangen, Germany), which automatically determined the borders of the tumor. In a second step, these borders were visually checked and, if needed, manually corrected. The software subsequently calculated the volume in which the mean attenuation value and the standard deviation of the attenuation value were determined. This enabled precise determination of the tumor volume even in case of an irregular shaped adrenal lesion. The standard deviation of each volumetric attenuation value was used as a measure of heterogeneity of the tumor. The amount of necrosis was estimated, and the presence of fine or coarse calcifications was graded.

The primary objective of this study was to determine the sensitivity of the 10 HU cut-off value to demonstrate or exclude the presence of a pheochromocytoma. Sensitivity was calculated as a percentage with 95% confidence intervals. As secondary objective of this study, we determined concordance between radiologists with respect to classification of the pheochromocytoma (i.e. attenuation ≤ or >10 HU) using Cohen's Kappa coefficient. Consistency of attenuation measurements between radiologists was assessed using the intraclass correlation coefficient (ICC) and a standard error of measurement. Consistency between the volumetric measurement and transversal measurement was assessed with ICC. Another, secondary objective of this study was to determine potential relationships between the attenuation value, presence of calcifications, tumor necrosis tumor heterogeneity and acquisition and reconstruction parameters using Pearson's or Spearman correlations coefficients were appropriate. Kruskal–Wallis and Mann– Whitney tests were used to assess the relationship between attenuation value and categorical data where appropriate. The difference between volumetric and transversal attenuation measurement was calculated as a marker of selection bias in the ROI placement. Plasma and urinary metanephrines were calculated as ratios of the upper reference limit maintained by each laboratory and the biochemical phenotype was determined as previously described (16).

Data are represented as mean± standard deviation (SD) or median (interquartile range) where appropriate. Statistical analyses were performed using SPSS, version 23.0 for Windows (IBM Corporation). A two-sided P value <0.05 was considered significant.

Results

We initially identified 224 eligible patients with pheochromocytoma from whom a preoperative unenhanced CT scan was available. After review, 10 cases were excluded since the pheochromocytoma was not fully imaged in nine cases and the image quality was insufficient in one case. Therefore, 214 patients harboring a total of 222 histologically confirmed pheochromocytomas were included into the final analysis. The mean age of patients was 53 ± 15 years, 60% were female and the biochemical phenotype was predominantly adrenergic (Table 1). In 32% of cases, the pheochromocytoma was diagnosed during the evaluation of an adrenal incidentaloma. Acquisition and reconstruction characteristics are shown in Table 2. In 89% of CT scans, tube potential was set at 120 kilovolt (kV) and in 85% of cases additional post- contrast series were available.

Table 1: Characteristics of 214 study subjects with a pheochromocytoma. Data are represented as mean± SD, median (IQR) or percentages.

Age (years)	53 ± 15
Sex (m/f)	40% / 60%
BMI (kg/m^2)	54.4 (21.8–27.9)
PCC localization (left/right/bilateral)	47% / 49% / 4%
Plasma MN (ratio of URL)	3.9 (1.1–14.3)
Plasma NMN (ratio of URL)	7.9 (2.3–18.8)
Urinary MN (ratio of URL)	4.1 (1.0–14.5)
Urinary NMN (ratio of URL)	3.6 (1.7–7.7)
Biochemical phenotype (A/NA)	66% / 34%

A, adrenergic; BMI, body mass index; MN, metanephrine; NA, noradrenergic; NMN, normetanephrine; PCC, pheochromocytoma; URL, upper reference limit.

Table 2: Acquisition and reconstruction parameters of 214 CT scans. Data are represented as percentage or median (IQR).

Parameters	**Values**
Slices	
16	17%
64	21%
320	14%
Other	28%
Unknown	20%
Slice thickness (mm)	3.0 (2.0–3.2)
Slice increment (mm)	2.0 (1.5–3.0)
Tube potential (kV)	
<120	7%
120	89%
>120	4%
Reconstruction kernel	
Soft	39%
Medium	56%
Unknown	5%

kV, kilovolt.

The maximum tumor diameter in any plane was 51 (39–74) mm and median tumor volume was 31 (13–92) cm^3. Mean unenhanced attenuation value within the ROI placed by each radiologist was 36 ± 9 HU and 37 ± 9 HU for observer 1 and observer 2, respectively (Table 3). The ICC was 0.81 (95% CI: 0.75–0.86) and standard error of measurement was 7.3 HU between observers. The unenhanced attenuation value of 221 pheochromocytomas was classified >10 HU by both radiologists, whereas one pheochromocytoma

was judged concordantly ≤10 HU. This resulted in perfect interobserver reliability: k = 1.00, P < 0.001. The sensitivity of the 10 HU cut-off for identifying a pheochromocytoma was 99.6% (95% CI: 97.5–99.9; Figs 1 and 2).

Figure 1: Bland–Altman plot of 222 pheochromocytoma showing the mean value of attenuation measurements by two radiologists from a single patient (X-axis) plotted against the difference between the same two results (Y-axis). The solid horizontal line represents the mean difference of all measurements with corresponding 95% limits of agreement represented as the dotted lines.

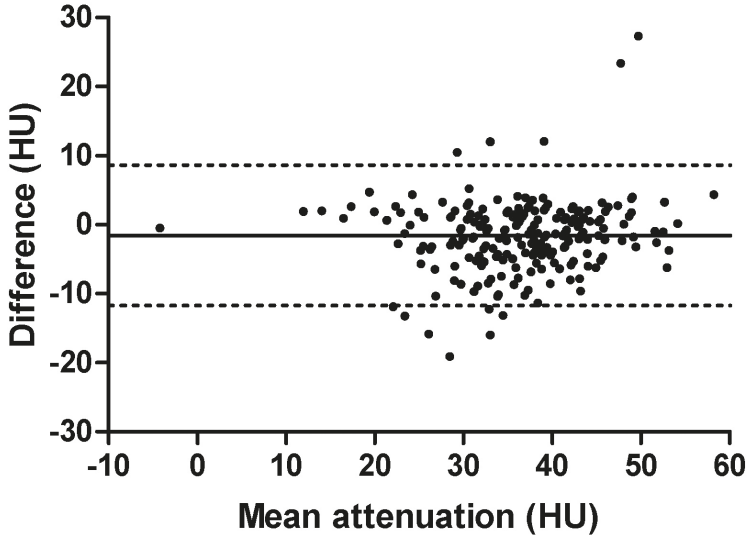

Figure 2: Cumulative distribution in percentage (Y-axis) displayed for the mean attenuation value (X-axis) for 222 pheochromocytomas on the left. On the right side, a magnification of the lower range of attenuation values including the intersection with the 10 HU cut-off value.

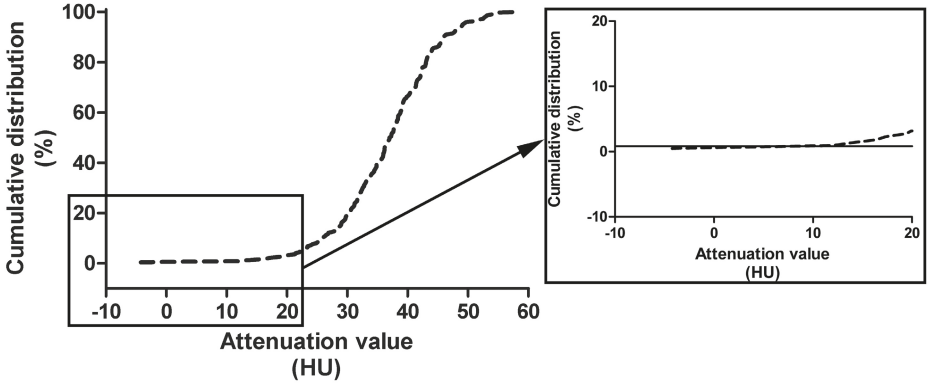

Table 3: Tumor characteristics and attenuation values of 222 pheochromocytomas. Data are represented as mean± SD, median (IQR) or percentages.

Tumor characteristics	Values	Observer 1	Observer 2	Volumetric
Diameter transversal plane (mm)	43 (33–64)			
Orthogonal diameter (mm)	36 (25–50)			
Maximum diameter (mm)	51 (39–74)			
Calcifications (%)				
None	93			
Fine	3			
Course	4			
Necrosis (%)				
0	51			
≤10	10			
11–50	21			
>50	18			
Region of interest (cm^2)*		4.8 (2.7–8.3)	2.9 (1.6–5.2)	31 (13–92)
Mean attenuation value (HU)			37 ± 9	32 ± 9
SD attenuation value (HU)		18 ± 7	18 ± 8	24 ± 9

*The region of interest for the volumetric measurement is expressed as cm^3. HU, Hounsfield Units.

Mean attenuation value in the VOI was 32 ± 9 HU. ICC was 0.75 (95% CI: 0.24–0.89) and standard error of measurement was 8.4 HU between the volumetric attenuation value and both measurements in the transversal plane. The difference between volumetric and transversal attenuation measurements was positively associated with tumor heterogeneity (standard deviation of the VOI attenuation value, R_S 0.23, P = 0.001) as well as, with the presence of necrosis (R_S 0.32, P < 0.001), but not with tumor volume (R_S 0.06, P = 0.399).

Interrater reliability between radiologists for scoring calcifications and necrosis was k = 0.56 and k = 0.54, respectively (P < 0.001 for both). The mean attenuation value in the transversal plane was not significantly associated with tumor volume, presence of calcifications, tube potential or tumor heterogeneity (data not shown). The mean attenuation value in the transversal plane was lower in tumors containing more than 50% necrosis (mean attenuation 37 ± 9, 38 ± 6, 38 ± 7 and 33 ± 7 HU for 0, 1–10, 11–50 and >50% necrosis respectively, P = 0.029).

The single patient with an unenhanced attenuation value ≤10 HU was found to have Cushing's syndrome caused by ectopic ACTH secretion from a left-sided pheochromocytoma. Thediagnosis ofpheochromocytoma was not considered

before surgery, but was established by pathological examination of the resected adrenal gland, demonstrating a 3.5 cm large tumor consisting of cell nests with eosinophilic cytoplasm, with positive immunohistochemical staining for chromogranine, synaptophysine, S-100 and ACTH. Preoperative urinary free cortisol excretion was 13 000 nmol/24 h. The unenhanced attenuation value measured by both radiologists was –4 HU and the VOI attenuation value was –15 HU. Remarkably, the standard deviation of the attenuation value of this tumor represented the highest value of the entire study population (i.e. 66 HU), despite the absence of necrosis, cystic parts or calcifications (Fig. 3).

Figure 3: Left-sided pheochromocytoma with ectopic ACTH production demonstrating an unenhanced attenuation value of –4 HU.

Discussion

In this large retrospective multicenter study, we show that the finding of an unenhanced attenuation value ≤10 HU in an adrenal gland tumor has a high diagnostic value of 99.6% to exclude the presence of a pheochromocytoma.

Moreover, the applicability of this method is underlined by the excellent interobserver agreement.

Over the past two decades, there has been a rapid increase in the utilization of various imaging techniques, which has led to a marked rise in the detection rate of adrenal incidentalomas. Radiological studies report a frequency varying from 3% to 10%, with the highest rates among the elderly (9, 17, 18, 19). The detection of an adrenal incidentaloma should be followed by further analysis in order to evaluate whether the lesion is hormonal active or not and whether the adrenal tumor is benign or malignant. For this purpose, several algorithms have been developed (7, 9, 20). One of the common denominators in all these algorithms is the recommendation to measure metanephrines in plasma or urine in order to exclude the presence of a pheochromocytoma. However, the recently issued ESE/ENSAT guideline has suggested that determination of metanephrines might be obviated in case of an adrenal incidentaloma with an unenhanced CT attenuation value ≤10 HU (9). This recommendation was based on small-sized studies predominantly describing pheochromocytomas with an unenhanced attenuation value >10 HU (21, 22). In agreement with these earlier reports, we demonstrate that pheochromocytomas with low unenhanced attenuation values are a very infrequent phenomenon.

Biochemical tests for pheochromocytoma have an excellent negative predictive value of 0.99 (23). However, the specificity depends quite strongly on preanalytical conditions, such as the use of certain drugs (e.g. tricyclic antidepressants, dopamine D2 receptor antagonists) and the requirement of a correctly collected 24-h urine or blood sampling after at least 20 min of supine rest for measurement of metanephrines in urine or plasma, respectively (24). In addition, determination of metanephrines is rather expensive. As it is not always feasible to create optimal preanalytical conditions the rate of false-positive measurements might be increased, subsequently resulting in more diagnostic testing to rule out pheochromocytoma at higher costs (25). Moreover, almost 70% of adrenal incidentalomas demonstrate attenuationvalues≤10 HU, illustratingt he large proportion of patients that might benefit from using the attenuation threshold in order to determine which patients need biochemical screening as second-line testing to rule out pheochromocytoma (26). Notably, approximately 3500 patients with adrenal incidentaloma and attenuation value ≤10 HU would need to be biochemically screened in order to diagnose one case of pheochromocytoma, assuming a 5% prevalence of pheochromocytoma in the adrenal incidentaloma

population, 70% frequency of attenuation ≤10 HU and a false-negative rate of 0.4% of radiological classification as determined in the present study. We cannot exclude, however, the possibility that under certain clinical circumstances determination of metanephrines might still be indicated in case of an adrenal mass with a attenuation <10 HU, e.g. in a patient with symptoms suggestive of a pheochromocytoma or when biopsy or adrenalectomy is considered.

The single patient with a false-negative CT scan had an unusual clinical picture of Cushing's syndrome due to ectopic ACTH production by a pheochromocytoma. In this case, the mean attenuation value might have been decreased as a result of the higher cholesterol content associated with the profound hypersecretion of cortisol as is also supported by the extremely high standard deviation of the attenuation value.

Our data of this measurement in daily practice is very reliable if basic rules of ROI drawing are taken into account (27). This is underlined by good interobserver consistency and excellent concordance between radiologists. Selective ROI placement was shown to be especially valuable in heterogeneous tumors with more necrosis. It has been shown that acquisition and reconstruction parameters of CT scans, such as tube potential and scanner type, can induce small changes in attenuation values and a lack of uniformity in these settings might be considered a shortcoming of the current study (28). For example, a significant proportion of adrenal incidentalomas was reclassified over time when using the 10 HU cut-off value due to variability between CT scanners and observer (29). However, the magnitude of this variability is very low and our data show that only a small proportion of pheochromocytomas demonstrates an attenuation value close to 10 HU. This suggests that differences in acquisition and reconstruction parameters or interobserver variability are unlikely to affect the radiologic classification of these adrenal tumors (28).

The major strengths of the current study are the systematic reassessment by two independent radiologists performing attenuation measurements in accordance with daily practice in a relatively large number of pheochromocytomas. A limitation of our study is the possibility of confirmation bias due to the non-blinded design or incorporation bias as CT is part of routine diagnostic work-up in case of a pheochromocytoma. Also, its retrospective design, and the fact that we were not able to determine the specificity of the HU threshold value further illustrate that clinical applicability since no control group was included might be considered as

a limitation. It should be noted, however, that the specificity of the 10 HU cut-off value is known to be very low for diagnosing a pheochromocytoma (30).

In conclusion, the likelihood that an adrenal incidentaloma with an unenhanced CT attenuation value ≤10 HU represents a pheochromocytoma is very low. Our results do not support the widespread clinical practice to determine metanephrines in every patient with an adrenal incidentaloma.

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Chapter 7

Diagnostic accuracy of CT imaging to exclude pheochromocytoma: a systematic review, meta-analysis and cost analysis

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Abstract

Objective: To assess the diagnostic accuracy of unenhanced CT attenuation to exclude a pheochromocytoma in the diagnostic work-up of patients with an adrenal incidentaloma and model the associated difference in diagnostic costs.

Methods: MEDLINE and EMBASE were searched from indexing up to September 27th, 2018 and studies reporting the proportion of pheochromocytomas on either side of the 10 HU threshold at unenhanced CT-scans were included. The pooled proportion of pheochromocytomas with an attenuation >10 HU was determined as well as the modelled financial costs of the current and alternative diagnostic approach. Registered under PROSPERO CRD42018097041.

Results: 2957 studies were identified. 31 studies were included, reporting on a total of 1167 pheochromocytomas. Overall risk of bias was low. Heterogeneity was not observed between studies (Q=11.5, *P*=.99, I^2 = 0.0%). The pooled proportion of patients with attenuation >10 HU was 0.990 (95% CI: 0.984-0.995). The modelled financial costs using the new diagnostic approach were €55 (~$63) lower per patient.

Conclusion: Pheochromocytomas can be reliably ruled out in case of an adrenal lesion with an unenhanced CT attenuation value ≤10 HU. Therefore, determination of metanephrines can be restricted to adrenal tumors demonstrating an unenhanced CT attenuation value >10 HU. Implementing this novel diagnostic strategy is cost-saving.

Introduction

An adrenal incidentaloma is an adrenal mass detected serendipitously on imaging studies performed for clinical reasons other than suspected adrenal disease (1). The prevalence of adrenal incidentalomas is estimated around 3% at the age of 50 years and increases up to 10% in 70- year-old patients (2). Overall, the chance of visualizing unexpected lesions such as adrenal incidentalomas has increased dramatically in recent years due to the exponential growth in the use of imaging techniques like ultrasonography, CT and MRI (3,4).

The key objectives in the analysis of a patient in whom an adrenal incidentaloma is detected are to establish whether or not hormonal hypersecretion or malignancy is present. Both biochemical tests and imaging procedures are performed to address these issues (1,2). In general, the adrenal incidentaloma population is characterized by a low prevalence of clinically important pathology, and it is estimated that over 70% of patients are eventually diagnosed with a benign, hormonally inactive, adrenal adenoma (5). However, it is mandatory to rule out the presence of a pheochromocytoma. If left untreated, a pheochromocytoma can result in severe cardiovascular morbidity and mortality (5-8). Therefore, it is currently recommended to determine metanephrines, the *O*-methylated metabolites of catecholamines, in plasma or in a 24-hour urine collection in every patient with an adrenal incidentaloma (1,2). This diagnostic strategy has several disadvantages. For instance, the specificity of the various assays used for measurement of metanephrines is significantly affected by preanalytic factors (6,9-12). As a result, suboptimal sampling conditions might increase the rate of false-positive measurements, necessitating additional diagnostic examinations and augmenting financial costs (13,14). Moreover, the measurement of metanephrines poses an additional burden to the patient either due to the requirement of blood sampling after 30 minutes of supine rest or the collection of a 24-hour urine sample (6).

Recently, we and others have proposed that extensive biochemical testing to rule out pheochromocytoma could be omitted in patients harboring an adrenal incidentaloma with an attenuation value ≤10 Hounsfield Units (HU) on unenhanced CT-scanning as this threshold was demonstrated to be highly sensitive (2,15). Such a strategy would not only be more patient-friendly, but also may considerably reduce the number of metanephrines measurements at no additional costs, as an unenhanced CT-scan is routinely performed in every patient harboring an adrenal incidentaloma in order to discriminate between benign (i.e. unenhanced

attenuation value ≤10 HU) and potentially malignant lesions (i.e. unenhanced attenuation value >10 HU) (1,2). This approach was also suggested in the latest ESE/ENSAT guideline, acknowledging, however, that definitive data in this area were lacking (2). Nonetheless, only a few studies with a relatively small sample size have reported on this topic casting doubt as to whether this diagnostic strategy is reliable enough to be implemented in clinical practice. We therefore decided to conduct a meta- analysis of studies describing unenhanced CT attenuation values of pheochromocytomas. Our primary aim was to determine the diagnostic accuracy of the 10 HU threshold value to exclude the presence of a pheochromocytoma. Our secondary aim was to determine the financial consequences of adopting the here presented novel diagnostic strategy.

Methods

Data Sources and Searches

In order to identify articles reporting unenhanced attenuation values of pheochromocytomas published in peer-reviewed medical journals, we conducted a systematic search of PubMed/MEDLINE and Embase, in agreement with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement (16). We used the following search terms: pheochromocytoma, adrenal incidentaloma, computed tomography, attenuation and synonyms for these terms (see Supplement for detailed information). The search was carried out on November 29th, 2017 and last updated on September 27th, 2018.

Study Selection

We considered articles to be eligible for inclusion if the proportion of pheochromocytomas with an average unenhanced attenuation value in a region of interest >10 and ≤10 HU could be extracted from the data. Language of the articles was restricted to English, German, French or Dutch. We excluded papers reporting on less than five pheochromocytomas. In case of overlapping study populations, the article describing the highest number of pheochromocytomas with available unenhanced attenuation values was selected or, where possible, original data was adjusted to correct for partial overlap.

Data Extraction and Quality Assessment

All titles and abstracts were independently reviewed by two investigators (EB and AB). Full-text articles were retrieved for potentially eligible cases. The reference lists of included articles were also screened for potentially eligible publications.

Both reviewers independently extracted data including study design, CT-scan protocol, attenuation measurement protocol, unenhanced attenuation values and demographics of the study participants. Discrepancies were solved based on consensus. All included articles were judged according to the Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) guidelines to assess the quality, risks of bias and, concerns regarding applicability to the current review's research question (17). A funnel plot was used to asses potential publication bias.

Data Synthesis and Analysis

The primary endpoint of this study was the pooled proportion of pheochromocytomas with an unenhanced attenuation value >10 HU. The secondary endpoint was the difference in laboratory costs between current practice, where metanephrines are routinely determined in every patient and an alternative strategy in which measurement of metanephrines would be exclusively performed in patients harboring an adrenal incidentaloma with an unenhanced attenuation value >10 HU.

Data are expressed as absolute or relative numbers, as mean (SD), or as a range. Heterogeneity was determined using the Cochran's Q-test and Higgins I^2. The weighted summary proportion of pheochromocytomas with an unenhanced attenuation value >10 HU and corresponding 95% confidence intervals were determined using a Freeman-Tukey transformation under the DerSimonian-Laird random-effects model (18). A sensitivity analysis was performed in a subset of studies that included only true adrenal incidentaloma populations. A second sensitivity analysis was performed in a subset of studies that included only CT-scans performed after the year 2000. The negative predictive value of an attenuation value ≤10 HU was calculated assuming a 5.6% prevalence of pheochromocytoma, the pooled proportion of pheochromocytomas with an unenhanced attenuation value >10 HU as determined in the current meta-analysis (i.e. reflecting the sensitivity), and a variable specificity. Additionally, the number of patients that harbor an adrenal incidentaloma with an unenhanced attenuation <10 HU that need to be biochemically screened to detect one pheochromocytoma was determined.

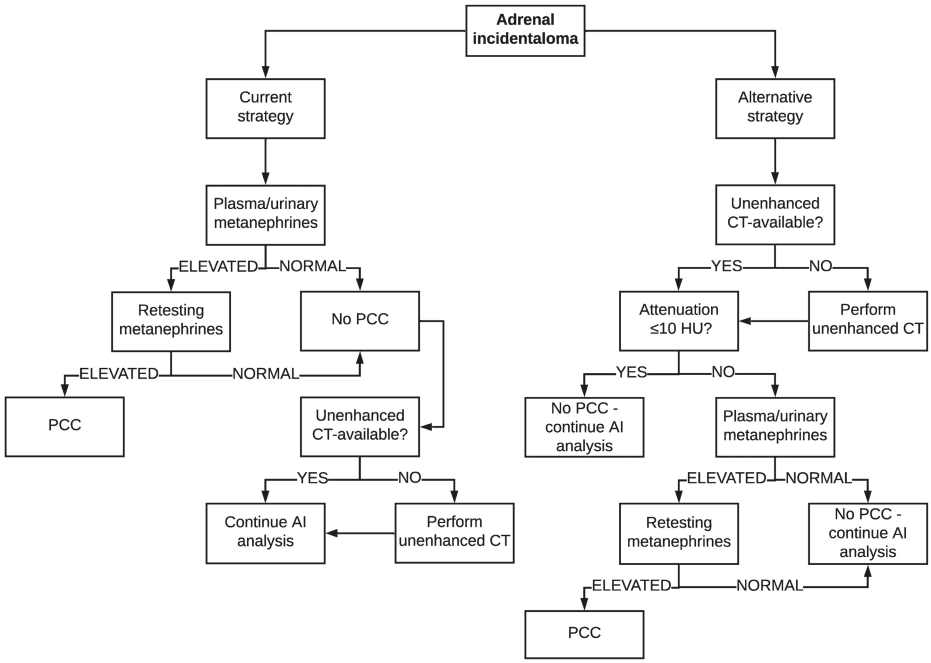
Review Manager 5.3 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) and MedCalc 18.2.1 (MedCalc Software, Ostend, Belgium) were used for statistical analysis.

Model-based Cost Study

We constructed a simple decision tree in Microsoft Excel 2010 (Microsoft

corporation, Redmond, WA, USA), reflecting the flow of the diagnostic workup in both the current and proposed strategy (Figure 1). The model simulated the diagnostic path of an average patient, applying both the current and the proposed novel strategy. The result of the model calculations was the total per patient diagnostic costs of each strategy from a healthcare perspective. We assumed a 5.6% (SEM: 2%) prevalence of pheochromocytoma in the adrenal incidentaloma population (5). Based on local review of CT-scans, it was estimated that on average 10% (range: 5-15%) of the CT- scans on which an adrenal incidentaloma is initially detected are performed without administration of intravenous radiocontrast (unpublished data). The frequency of unenhanced attenuation values ≤10 HU was determined to be 69% (SEM: 10%) based on a weighted average of previously reported frequencies (19-21). In this model we opted for plasma metanephrines as this is the most accurate and patient-friendly method for determination of metanephrines (14,22). The results of plasma metanephrines were predicted using a sensitivity and specificity of 98.6% and 95.1%, respectively (22,23). These test characteristics were previously determined under optimal preanalytical conditions. Retesting of patients with plasma concentrations of metanephrines above the upper reference limit was included since borderline elevated metanephrines are not uncommon. In the model retesting was considered to be a second determination of plasma metanephrines and was applied in 38% (SEM: 5%) of cases with elevated metanephrines as previously demonstrated (24). The financial costs of a single plasma metanephrines measurement and an unenhanced CT-scan of the abdomen is €78 ($89) and €194 ($221), respectively, according to the 2018 Dutch Healthcare Authority rates (25). Apart from a deterministic analysis based on the point estimates of all input parameters, we also performed a probabilistic sensitivity analysis (PSA) to assess the impact of the joint uncertainty in the input parameters. The PSA generates 1,000 estimates of results, based on random values of the input parameters, drawn from distributions around the point estimates. We assumed that other diagnostic tests that are usually performed in case of an adrenal incidentaloma to rule out hypercortisolism, primary aldosteronism and malignancy would be equally applied using both strategies. Therefore these costs were not included in the current analysis. The cost study was, where applicable, performed and reported according to the Consolidated Health Economic Evaluation Reporting Standards (CHEERS) (26).

Figure 1: Economic evaluation flowchart of the currently recommended and the alternative strategy.



Results

Included Studies

The primary search yielded a total of 2957 articles. After removing duplicates and screening abstracts 208 full-text articles were evaluated for eligibility, out of which 31 articles were included in the meta-analysis (Supplemental Figure 1). Of note, the study from Canu et al.(27) had partially overlapping study populations with studies from Buitenwerf et al.(15), Iñiguez-Ariza et al.(28) and Marty et al.(21) for which we corrected by excluding duplicates (n=98) from the first study.

The main characteristics of all included studies are presented in Table 1. (15, 20, 21, 27, 29-54). Twenty- nine out of 31 included studies were retrospective and the majority were not primarily designed to evaluate the diagnostic utility of an unenhanced CT to exclude the presence of a pheochromocytoma.

Table 1. Characteristics of included studies.

Author	Year	Country	Total population	№ PCC patients	№ PCC	№ unenhanced CT	ROI placement	Tube voltage (kV)	№ observers	Reference test
Adams [29]	1983	UK	73	9	9	9	unknown	unknown	unknown	PA
Neumann [30]	1984	Germany	14	11	11	11	unknown	unknown	unknown	PA
Nicolas [31]	1985	Germany	29	6	6	5	unknown	unknown	unknown	PA
Miyake [32]	1989	Japan	36	8	8	8	unknown	unknown	unknown	PA
Saginoya [33]	2001	Japan	29	19	19	19	unknown	unknown	unknown	PA
Blake [34]	2003	USA	9	8	8	8	yes	140	2	PA
Szolar [20]	2005	Austria	67	17	17	17	yes	120	2	PA
Kasperlik-Zaluska [35]	2006	Poland	1111	33	33	33	unknown	unknown	unknown	PA
Bessell-Browne [36]	2007	Canada	25	12	16	16	unknown	unknown	unknown	PA
Park BK [37]	2007	South-Korea	53	53	60	31	yes	120	2	PA
Park SH [38]	2007	South-Korea	45	12	12	12	yes	120	1	PA
Ctvrlik [39]	2009	Czech Republic	55	9	9	9	yes	unknown	unknown	PA
Hong [40]	2011	South-Korea	187	19	19	19	unknown	unknown	unknown	unknown
Foti [41]	2012	Italy	104	5	5	5	yes	120	2	PA
Sane [42]	2012	Finland	184	9	10	9	yes	unknown	unknown	PA
Park SW [43]	2012	South-Korea	244	48	48	48	yes	120	2-3	PA
Pitts [44]	2013	USA	243	10	10	10	unknown	unknown	unknown	PA
Raja [45]	2013	Canada	53	53	58	41	unknown	120	1	PA
Angelelli [46]	2013	Italy	50	7	7	7	unknown	120	2	PA
Shawa [47]	2014	USA	32	9	9	9	unknown	unknown	1	PA
Patel [48]	2014	USA	124	42	47	37	yes	100-140	3	PA or MIBG

Table 1: Continued

Author	Year	Country	Total population	№ PCC patients	№ PCC	№ unenhanced CT	ROI placement	Tube voltage (kV)	№ observers	Reference test
Kannan [49]	2014	USA	320	112	112	108	unknown	unknown	2	PA
Jun [50]	2015	South-Korea	234	19	19	19	yes	unknown	1	PA or MN
Zhu [51]	2016	China	110	14	14	14	yes	120	unknown	PA
Schieda [52]	2016	Canada	32	32	34	10	yes	120	1	PA
Buitenwerf [15]	2017	The Netherlands	214	214	222	222	yes	variable	2	PA
Ohno [53]	2018	Japan	297	21	21	21	yes	unknown	unknown	PA
Marty [21]	2018	France	233	33	33	33	yes	120	2	PA or MN
Iñiguez-Ariza [28]	2018	USA	705	158	171	63	yes	unknown	1	PA
Dineen [54]	2018	Ireland	208	36	36	36	unknown	unknown	unknown	PA
Canu [a] [27]	2018	International	435	435	450	278	no	unknown	unknown	PA
Overall			**5555**	**1473**	**1533**	**1167**				

[a] numbers after adjustment of partially overlapping populations.
Data are expressed as absolute numbers. PCC: pheochromocytomas, CT: computed tomography, ROI: region of interest, kV: kilovolt, PA: histopathology. MN: metanephrines, MIBG: ^{123}I-metaiodobenzylguanidine.

Study Quality and Risk of Bias

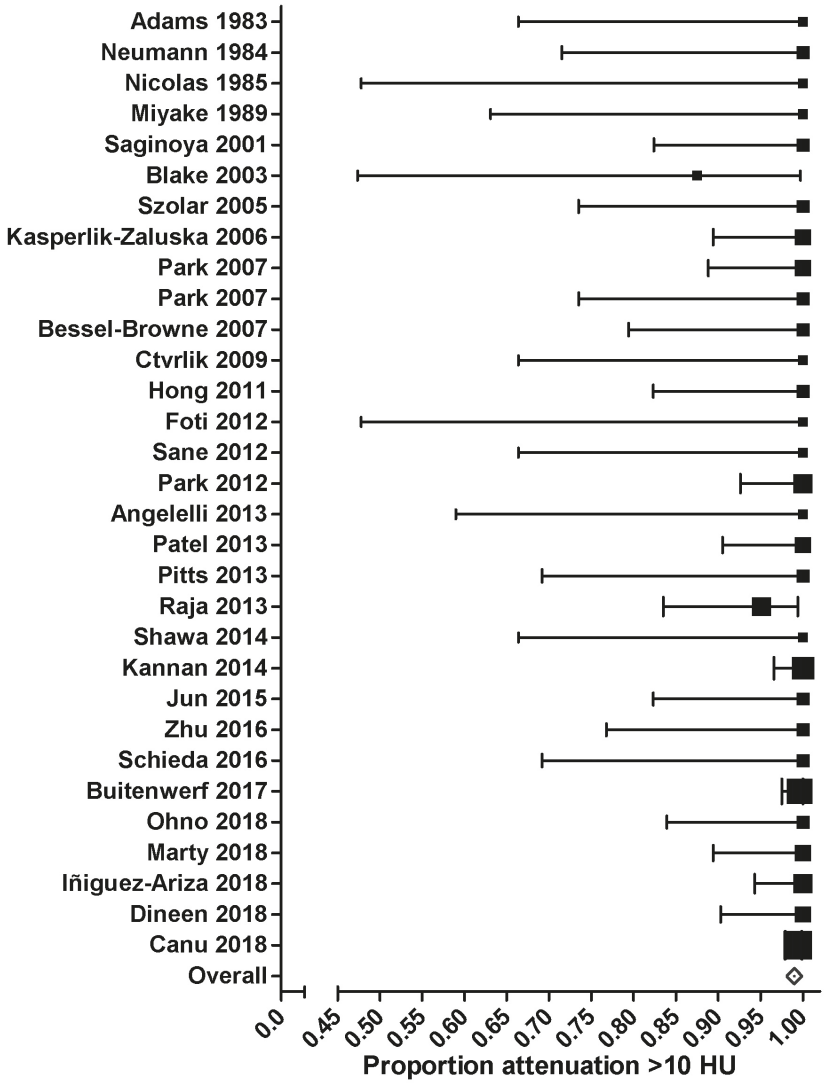
Based on the QUADAS-2 tool, the overall risk of bias and concern for applicability were considered to be low for the majority of the included studies (Supplemental Figure 2 and Supplemental Table 1). The risk of selection bias was considered to be low because most studies enrolled a consecutive or random sample of pheochromocytoma patients and avoided inappropriate exclusions. In two studies the selection procedure of pheochromocytoma patients was not clearly described (47,54). Unenhanced CT scanning was not performed in all reported patients with a pheochromocytoma, which reflects routine clinical practice and is unlikely to reflect systematic bias. Technical specifications of the unenhanced CT-scan were often sparsely reported. For example, acquisition and reconstruction parameters were not standardized in 77% of studies due to their predominant retrospective nature. Of note, tube voltage is recognized as a significant determinant of attenuation and was set at 120 kV in 32% of the studies (55). Therefore, variation in the index test was identified as a significant risk of bias. The drawing of the region of interest, in which the attenuation value was determined, was adequately described in 45% of studies and in 32% performed by two or more radiologists. Histopathological proof of pheochromocytoma was the single-used reference standard in 27 studies. There were a total of four pheochromocytomas in three studies for which either biochemistry (n=3) or ^{123}I- metaiodobenzylguanidine (MIBG) scintigraphy (n=1) was used as a reference standard instead of histopathology (21,48,50). The reference standard was not described in one study (40). The funnel plot was completely symmetrical indicating that publication bias was unlikely (Supplemental Figure 3).

Diagnostic Accuracy

The 31 included studies collectively reported on a total of 1533 pheochromocytomas. Unenhanced CT-scan was performed in 1167 confirmed pheochromocytoma cases, of which 1161 pheochromocytomas had an unenhanced attenuation value >10 HU and six had an unenhanced attenuation value ≤10 HU. The proportion of patients with an unenhanced attenuation >10 HU ranged from 0.875 (95% CI: 0.474 - 0.997) to 1.00 (95% CI: 0.966 – 1.000) in each individual study (Table 2, Figure 2). Heterogeneity was not observed between studies (Q=11.5, P=.99, I^2 = 0.0%). The pooled proportion of patients with an unenhanced attenuation >10 HU was 0.990 (95% CI: 0.984-0.995).

The sensitivity analysis in a subset of 14 studies reporting on 828 pheochromocytomas of which the CT-scans were performed after the year 2000 demonstrated a pooled proportion of unenhanced attenuation >10 HU of 0.990 (95% CI:0.982-0.996). (15,21,27,28,38,39,41-43,48,50-53) The second sensitivity analysis included 6 studies that reported 120 pheochromocytomas from true adrenal incidentaloma populations. (21,35,40-42,53) The pooled proportion in this analysis was 0.989 (95% CI:0.963-0.999).

Figure 2: Forest plot demonstrating the proportion of pheochromocytomas with an unenhanced attenuation value >10 Hounsfield Units with 95% confidence inter-vals.



The proportional negative predictive value of an attenuation value ≤10 HU was close to 1 for a wide range of specificity values (Figure 3). It was calculated that 1232 patients harboring an adrenal tumor with an unenhanced attenuation value <10 HU needed to be biochemically screened to detect one pheochromocytoma assuming a 5.6% prevalence and 69% frequency of an attenuation value <10 HU among adrenal incidentalomas.

Table 2. Diagnostic results of unenhanced CT-scanning in patients with a pheochromocytoma.

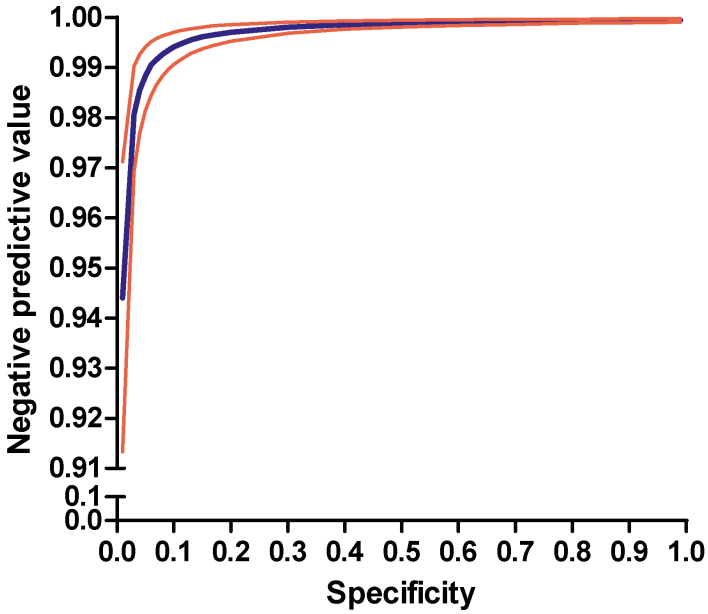
Author	Mean attenuation value (SD)	Range of attenuation (HU)	False negatives (№)	True positives (№)	Proportion >10 HU
Adams [29]	-		0	9	1.00 (0.664-1.00)
Neumann [30]	50	33-80	0	11	1.00 (0.715-1.00
Nicolas [31]	-	25-60	0	5	1.00 (0.478-1.00)
Miyake [32]	36 (10)	21-50	0	8	1.00 (0.631-1.00)
Saginoya [33]	35	15-50	0	19	1.00 (0.824-1.00)
Blake [34]	-		1	7	0.875 (0.474-0.997)
Szolar [20]	44 (11)	28-60	0	17	1.00 (0.735-1.00)
Kasperlik-Zaluska [35]	-	All > 25	0	33	1.00 (0.894-1.00)
Bessell-Browne [36]	41	23-53	0	16	1.00 (0.794-1.00)
Park BK [37]	41 (10)	19-58	0	31	1.00 (0.888-1.00)
Park SH [38]	40 (6)	37-49	0	12	1.00 (0.735-1.00)
Ctvrlik [39]	40 (9)	25-60	0	9	1.00 (0.664-1.00)
Hong [40]	32 (9)		0	19	1.00 (0.823-1.00)
Foti [41]	27 (7)	24-38	0	5	1.00 (0.478-1.00)
Sane [42]	-	25-67	0	9	1.00 (0.664-1.00)
Park SW [43]	29	21-78	0	48	1.00 (0.926-1.00)
Pitts [44]	33	20-42	0	10	1.00 (0.692-1.00)
Raja [45]	37 (8)		2	39	0.951 (0.835-0.994)
Angelelli [46]	30 (9)	14-44	0	7	1.00 (0.590-1.00)
Shawa [47]	38	20-51	0	9	1.00 (0.664-1.00)
Patel [48]	38	15-54	0	37	1.00 (0.905-1.00)
Kannan [49]	-	All > 17	0	108	1.00 (0.966-1.00)
Jun [50]	40	26-42	0	19	1.00 (0.823-1.00)
Zhu [51]	-		0	14	1.00 (0.768-1.00)
Schieda [52]	36 (7)	24-48	0	10	1.00 (0.692-1.00)

Table 2. Continued

Author	Mean attenuation value (SD)	Range of attenuation (HU)	False negatives (№)	True positives (№)	Proportion >10 HU
Buitenwerf [15]	37 (9)		1	221	0.996 (0.975-1.00)
Ohno [53]	37	32-43	0	21	1.00 (0.839-1.00)
Marty [21]	34 (9)	17-53	0	33	1.00 (0.894-1.00)
Iñiguez-Ariza [28]	33	18-60	0	63	1.00 (0.943-1.00)
Dineen [54]	28 [15-30] [a]		0	36	1.00 (0.903-1.00)
Canu [b] [27]	35 (10)		2	276	0.993 (0.974-0.999)
Overall			**6**	**1161**	**0.990 (0.984-0.995)**

The proportion >10 HU is presented as proportion with 95% confidence interval. HU: Hounsfield units. [a] median [interquartile range] [b] numbers after adjustment of partially overlapping populations

Figure 3: Negative predictive value of the 10 Hounsfield Units threshold value to exclude a pheochromocytoma (Y-axis) according to specificity (X-axis) and assuming a sensitivity of 0.990 (blue line) with 95% confidence interval 0.984 - 0.995 (red lines) and a 5.6% prevalence of pheochromocytomas.



A total of six pheochromocytomas with an attenuation value ≤10 HU were identified in four studies (15,27,34,45). The exact attenuation values of these cases were -4, 6, 9, 9, 10 and, 10 HU. In three cases the histopathological examination identified significant cystic, hemorrhagic or necrotic parts that could potentially have resulted in a decrease of the CT-attenuation value (27,45). In one case the histopathological examination described ''abundant fatty cytoplasm'' in intermingled cells (34). Another case involved a pheochromocytoma with ectopic ACTH production causing Cushing's syndrome (15). In addition, there was one case demonstrating concomitant cortical multinodular hyperplasia (27).

Cost Analysis

Deterministic analysis showed that the cost of the currently recommended strategy was €255 ($291) compared to €200 ($228) using the novel strategy with a cost difference between strategies of €55 ($63) on a per patient basis. In the probabilistic sensitivity analysis, costs were €255 ± 5 ($255 ± 6) compared to €200 ± 10 ($228 ± 11) for the current and novel diagnostic strategies, respectively. The cost difference was €55 ± 8 ($63 ± 9) and always in favor of the novel strategy,

ranging from €26 ($30) to €73 ($83) (Supplemental Figure 4). By definition there will be more biochemical tests in the current diagnostic strategy, while the number of CT-scans is almost equal between strategies. Therefore, the new strategy is cost saving at all price levels of CT-scans and metanephrines (data not shown).

Discussion

In the current meta-analysis we have demonstrated that a diagnosis of pheochromocytoma is highly unlikely in case of an adrenal tumor with an attenuation value ≤10 HU on an unenhanced CT-scan. This finding has important clinical consequences, as it implies that measurement of metanephrines could be restricted to adrenal masses with an attenuation value >10 HU. Adopting this new strategy for the evaluation of an adrenal incidentaloma is cost saving and also more patient friendly.

The clinical paradigm to always exclude a pheochromocytoma in case of an adrenal incidentaloma is based on the premise that such a diagnosis should never be missed, since this can result in severe cardiovascular morbidity and mortality (6,7). Therefore, determination of metanephrines is currently recommended in every patient with an adrenal incidentaloma regardless of the CT attenuation value (1,2). The present meta-analysis, comprising a large number of pheochromocytomas derived from various populations with an adrenal tumor, offers an alternative strategy by demonstrating a high diagnostic accuracy of the 10 HU threshold value to exclude a pheochromocytoma. A sensitivity analysis of only true adrenal incidentaloma populations and studies using modern CT-scanners yielded similar results. Therefore, it seems clinically advantageous to preselect patients for biochemical testing according to the unenhanced attenuation value. From the literature, it can be estimated that approximately 69% of adrenal incidentalomas demonstrate an attenuation value ≤10 HU on unenhanced CT- scan (19,21,42). Thus, omitting the measurement of metanephrines in this subgroup would affect a large proportion of patients. Notably, this alternative strategy would not result in more CT-scans, since an unenhanced CT is recommended in every patient with an adrenal incidentaloma in order to differentiate between a benign and a possibly malignant lesion (2). In addition, the strategy we propose would allow for optimal utilization of the superior specificity of metanephrines by significantly increasing the pretest probability for the presence of a pheochromocytoma.

Moreover, pre-analytical factors that are known to influence the diagnostic

accuracy of metanephrine assays, such as the use of certain drugs, co-morbidities, posture during blood sampling or incorrect collection of 24-hour urine, only need to be optimized in a smaller proportion of patients when using the proposed strategy (6,9-12). This would improve patient convenience and contribute to a reduction in the absolute number of false-positive metanephrine results and subsequent need for retesting. It seems, however, reasonable to still determine metanephrines in case adrenal biopsy or surgery is considered, regardless of the CT attenuation value.

Specificity of unenhanced CT-scanning could not be determined in the current analysis since the majority of the included studies suffered from selection bias and did not represent an unselected population with an adrenal incidentaloma. For instance, lipid-poor adenomas and malignancies such as adrenocortical carcinomas and metastases were often overrepresented in the studies we evaluated. These tumors are usually characterized by attenuation values >10 HU, which would have resulted in a falsely decreased specificity of the CT cut-off value. In addition, several studies included only pheochromocytomas, which obviously precluded determination of the specificity. In line with the observation that unenhanced attenuation values of pheochromocytomas overlap with adrenal tumors of another etiology, the specificity of unenhanced CT-scanning should be regarded to be suboptimal. However, we have demonstrated that the proportional negative predictive value of an unenhanced attenuation value ≤10 HU is close to 1, regardless of the specificity. These findings strongly support our hypothesis that a pheochromocytoma can be reliably excluded when the unenhanced CT-attenuation value is ≤10 HU. Moreover, we have previously demonstrated that the interrater reliability for measurement of attenuation values in adrenal tumors is excellent, facilitating correct radiologic classification in daily clinical practice (15). Of note, other radiological characteristics such as assessment of radiocontrast wash-out on CT have also demonstrated insufficient sensitivity for this purpose (27,56).

The clinical consequences of missing a pheochromocytoma can be severe, although the risk of a false-negative radiological classification is small. The number needed to screen to find one pheochromocytoma is very high when metanephrines are determined in all patients with an adrenal incidentaloma demonstrating an attenuation value <10 HU. Moreover, the actual number needed to screen is probably much higher than 1232, as the assumed prevalence of pheochromocytomas among patients with an adrenal incidentaloma is most likely overestimated due to selection bias by reports from central referral hospitals.

Nonetheless, clinicians should be aware of a few potential caveats. The main cause of radiological misclassification is the unrecognized presence of necrotic, hemorrhagic or cystic parts in a pheochromocytoma which are erroneously included in the region of interest during assessment of the attenuation value. In three out of six pheochromocytomas with a false-negative CT-scan the histopathological examination demonstrated necrosis, hemorrhagic or cystic parts (27,45). Post-contrast series aid in the identification of areas that should not be incorporated into the region of interest and might, therefore, prevent this type of misclassification. It is important that the radiologist follows the basic rules for drawing the region of interest in which the attenuation value is assessed (57).

We estimated that the here presented alternative diagnostic strategy for the evaluation of an adrenal incidentaloma will result in a modest reduction in financial costs per patient. It should be noted that the budget impact model provides a conservative cost saving estimate. In view of a 3 to 10% prevalence of adrenal incidentalomas and the exponential growth in the use of imaging studies, the total annual cost savings at the population level are likely to become substantial when adopting the proposed diagnostic strategy (3,58). Generalizability of these estimates depends mainly on local reimbursement rates for performing an unenhanced CT-scan and measurement of metanephrines. However, the alternative strategy remained cost saving in our model, even in a scenario with increased costs for CT and reduced costs for biochemical testing. An interactive version of the model is accessible through the website for reference.

Some limitations of the current study need to be discussed. We were not able to retrieve absolute attenuation values but only the proportions of pheochromocytomas on either side of the 10 HU cut-off value. Therefore, the optimal attenuation threshold value to exclude the presence of a pheochromocytoma could not be established. The lack of absolute attenuation values also hampered identification of borderline cases that were potentially at risk for misclassification due to heterogeneity in acquisition and reconstruction parameters or interobserver variability. However, we have previously demonstrated that this variation is unlikely to affect radiological classification as most pheochromocytomas have unenhanced attenuation values well above the 10 HU threshold.(15) In support of this, the lowest unenhanced CT attenuation values reported in the majority of the studies included in our meta-analysis were well above the 10 HU threshold. Therefore, the clinical relevance of this variation seems to be minor and, in our opinion, the observed heterogeneity in CT-scan settings

underscores the general clinical applicability of the proposed new diagnostic strategy. It would be desirable to evaluate the alternative strategy we here describe in a prospective study.

In conclusion, pheochromocytoma can be reliably ruled out when the unenhanced CT attenuation value of an adrenal tumor is ≤10 HU. As a result, determination of metanephrines can be restricted to adrenal tumors with an unenhanced CT attenuation value >10 HU. Implementing this novel diagnostic strategy is likely to be more patient-friendly and could reduce diagnostic costs.

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Supplemental data

Search strategies for the meta-analysis:

Pubmed

("Pheochromocytoma"[Mesh] OR "Adrenal incidentaloma" [Supplementary Concept] OR pheochromocytoma*[tiab] OR pheochromocytoma*[tiab] OR Adrenal incidentaloma*[tiab]) AND ("Tomography, X-Ray Computed"[Mesh] OR computed tomograph*[tiab] OR CT[tiab]) AND ("Pheochromocytoma/diagnostic imaging"[Mesh] OR hounsfield[tiab] OR hu[tiab] OR density[tiab] OR attenuation[tiab] OR enhancement*[tiab])

Embase

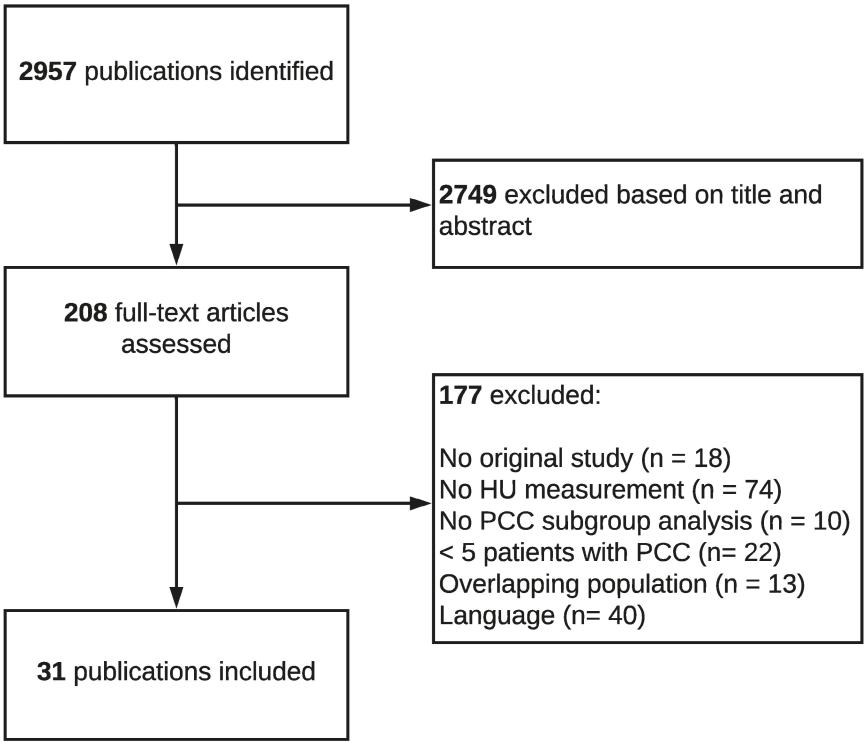
('pheochromocytoma'/exp OR 'adrenal incidentaloma'/exp OR (pheochromocytoma* OR pheochromocytoma* OR 'Adrenal incidentaloma*'):ab,ti) AND ('computer assisted tomography'/exp OR ('computed tomograph*' OR CT):ab,ti) AND ('pheochromocytoma'/exp/mj/dm_di OR (hounsfield OR hu OR density OR attenuation OR enhancement*):ab,ti)

Supplemental Table 1: Quality Assessment of Diagnostic Accuracy Studies - 2 (QUADAS-2) scores

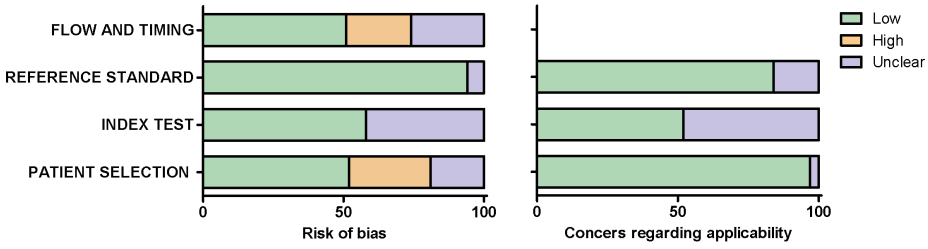
Author	Risk of Bias				Applicability concerns		
	Patient selection	Index test	Reference standard	Flow and timing	Patient selection	Index test	Reference standard
Adams	low	unclear	low	high	low	unclear	unclear
Neumann	high	unclear	low	low	low	unclear	low
Nicolas	high	unclear	low	unclear	low	unclear	low
Miyake	unclear	unclear	low	low	low	unclear	low
Saginoya	unclear	unclear	low	unclear	low	unclear	low
Blake	low	low	low	low	low	low	low
Szolar	low	low	low	high	low	low	unclear
Kasperlik-Zaluska	low	unclear	low	high	low	unclear	low
Bessell-Browne	low	unclear	low	low	low	unclear	low
Park BK	high	low	low	low	low	low	low
Park SH	low	low	low	low	low	low	low
Ctvrlik	low	low	low	low	low	unclear	low
Hong	unclear	unclear	unclear	high	low	unclear	unclear
Foti	high	low	low	unclear	low	low	low
Sane	low	low	low	low	low	unclear	low
Park SW	low	low	low	low	low	low	low
Pitts	high	unclear	low	unclear	low	unclear	low
Raja	low	unclear	low	low	low	low	low
Angelelli	unclear	low	low	low	unclear	low	low
Shawa	high	unclear	low	unclear	low	unclear	low
Patel	low	low	unclear	high	low	low	unclear
Kannan	low	low	low	low	low	unclear	low
Jun	low	low	low	high	low	unclear	low
Zhu	unclear	low	low	high	low	low	unclear
Schieda	low	low	low	low	low	low	low
Buitenwerf	low	low	low	low	low	low	low
Ohno	low	low	low	unclear	low	low	low
Marty	high	low	low	low	low	low	low
Iñiguez-Ariza	high	low	low	low	low	low	low
Dineen	unclear	unclear	low	unclear	low	low	low
Canu	high	unclear	low	unclear	low	unclear	low

Supplemental figures

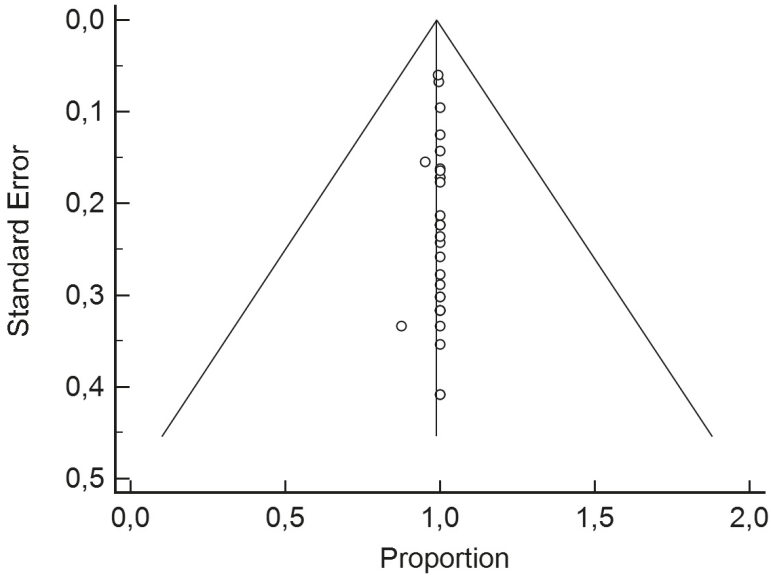
Supplemental Figure 1: Flowchart of included studies.



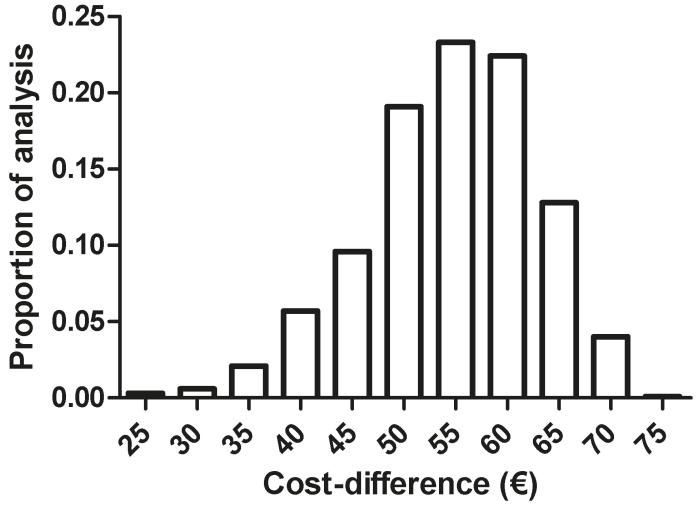
Supplemental Figure 2: Risk of bias (left) and concerns for applicability (right) of included studies using QUADAS-2.



Supplemental Figure 3: Funnel plot of included studies.



Supplemental Figure 4: Histogram showing the cost difference between the cur-rent and novel diagnostic strategy to exclude a pheochromocytoma in case of an adrenal incidentaloma as calculated in a probabilistic sensitivity analysis.



PART III

Adrenal medulla: optimizing perioperative hemodynamic stability in PPGL - The PRESCRIPT study

Chapter 8

The haemodynamic instability score: Development and internal validation of a new rating method of intra-operative haemodynamic instability

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Abstract

Background: There is no consensus on how to define haemodynamic instability during general anaesthesia. Patients are often classified as stable or unstable based solely on blood pressure thresholds, disregarding the degree of instability. Vasoactive agents and volume therapy can directly influence classification but are usually not considered.

Objective: To develop and validate a scoring tool to quantify the overall degree of haemodynamic instability.

Design: Retrospective observational study.

Setting: University hospital.

Patients: The development cohort consisted of 50 patients undergoing high-risk surgery with a control group of 50 undergoing video-assisted thoracoscopic surgery. In the validation cohort, there were 153 high-risk surgery patients and 78 controls.

Intervention: None.

Main Outcome Measures: The haemodynamic instability score (HI-score) was calculated as a weighted continuous measure ranging from 0 to 160 points, intended to reflect deviations of blood pressure and heart rate from predefined thresholds, and infusion rates of vasoactive agents and fluids. Thresholds were first determined in a development cohort and subsequently tested in a validation cohort. Results are presented as median [interquartile range].

Results: In the validation cohort the HI-score was 59 [37 to 96] in the high-risk surgery group compared with 44 [24 to 58] in the control group ($P < 0.001$). The score of the haemodynamic domain did not differ ($P = 0.69$) between groups: 10 [8 to 16] vs. 10 [8 to 16]. However, scores for volume therapy and vasoactive medication were significantly higher in the high-risk surgery group compared with the control group: 14 [6 to 30] vs. 6 [2 to 18], $P = 0.003$ and 35 [15 to 75] vs. 15 [5 to 35], $P < 0.001$, respectively.

Conclusion: We developed the HI-score and demonstrated that it can appropriately quantify the degree of intra-operative haemodynamic instability. The HI-score provides a clinical tool which, after further external validation, may have future applications in both patient management and clinical research.

Introduction

One of the key objectives of general anaesthesia is to maintain haemodynamic stability during surgery. This objective may be affected by many factors, such as pharmacodynamic effects of anaesthetic drugs on vascular tone and cardiac function, volume shifts or intra-operative hypothermia or hyperthermia. Haemodynamic stability is generally maintained with either volume therapy or vasoactive medication. The reported association of intra-operative hypotension and hypertension with postoperative morbidity and mortality suggests that haemodynamic stability is important for patient outcome (1–5).

In clinical reports haemodynamic instability has usually been described as a dichotomous variable, classifying a patient as either stable or unstable. There is, however, a lack of consensus on the definition of haemodynamic instability. In a meta-analysis of 46 studies there were 20 different dichotomous definitions of haemodynamic instability, predominantly based on absolute blood pressure (BP) thresholds (6). Since haemodynamic instability reflects a variety of conditions it seems more reasonable to define haemodynamic instability as a continuous spectrum ranging from stable to extremely unstable. In addition, the interventions required to restore stability, for example the administration of vasoactive agents, can directly affect the classification of a patient as stable or unstable when using a dichotomous definition. Moreover, there is an association between the amount of vasoactive agents and fluids given and morbidity and mortality (7,8). Therefore, both the haemodynamic variables and also the therapeutic interventions aimed at their stabilisation should be taken into account when assessing the overall degree of haemodynamic instability, particularly since the interventions might keep the variables within the normal range.

The aim of this study was to develop and validate a descriptive score to quantify the overall degree of haemodynamic instability during general anaesthesia. Such a score could be useful for clinical assessment of the degree of haemodynamic instability during a surgical procedure and identification of predictors for haemodynamic instability. Furthermore, a quantitative clinical score would also offer the possibility of a comparison between the efficacy of different interventions used to prevent and correct haemodynamic instability during general anaesthesia.

Methods

The current retrospective study had approval waived by the medical research and ethics

committee of the University of Groningen, The Netherlands, according to the Dutch Medical Research Involving Human Subjects Act. It was conducted at the University Medical Center Groningen in the Netherlands between January 2014 until October 2017.

We developed and validated a scoring system describing the degree of intra-operative haemodynamic instability, called the Haemodynamic Instability-score (HI-score). Patients covering a broad haemodynamic spectrum from stable to very unstable were included to determine normalised threshold values for all the components of the HI-score and, subsequently, to validate the HI-score by testing its ability to quantify haemodynamic instability in a separate internal validation cohort. Intra-arterial continuous BP monitoring was required in all patients for accurate determination of the HI-score. We therefore chose to use patients undergoing video-assisted thoracoscopic surgery as the control group since this type of surgery was expected to reflect a low degree of haemodynamic instability while still requiring an arterial cannula. This was primarily intended for blood gas analysis, but was also used for continuous BP monitoring. To reflect a high degree of haemodynamic instability we chose patients undergoing high-risk abdominal surgery (HRS) (9). Cardiac output monitoring to facilitate haemodynamic optimisation was part of standard patient care for high-risk surgery using the FloTrac/EV1000 system (Edwards Lifesciences, Irvine, California, USA). We included patients aged at least 18 years and excluded those who had received inotropic or vasoactive medication that was not routinely used in our hospital.

Data were extracted from the hospital patient data management system (PDMS) which accurately records haemodynamic variables and the administration of medication and fluids. We retrieved the following variables during the interval between incision and the end of surgery: heart rate (HR), mean arterial pressure (MAP), systolic arterial pressure (SAP), intravenously administered vasoactive medication, volume therapy including blood transfusions, and the duration of the procedure. BP and HR recordings were collected at intervals of 15 s. The anaesthetic induction period was omitted since in many patients the arterial cannula was placed after induction preventing incorporation of this data into the score. Baseline characteristics of all patients were extracted from the electronic patient charts.

Haemodynamic instability score development

The HI-score was developed using data from 50 HRS and 50 control patients who were randomly selected from the entire cohort. The design of the HI-score was based on two main criteria. First, it should not only encompass haemodynamic

variables like BP and HR, but also interventions with a direct effect on haemodynamic stability. Second, each component of the HI-score must be part of the routine measurements performed during general anaesthesia for surgical procedures with a certain risk of haemodynamic instability. Therefore, we chose the following three domains as part of the HI-score: haemodynamic variables (SAP, MAP, HR), intravenous volume therapy, and intravenous administration of vasoactive medication. Each domain was scored separately on a semiquantitative scale reflecting either the degree by which each variable deviated from the predefined threshold value or from the distribution of measurements in the development cohort as described below. Separate scores of the three domains were subsequently added up to form a total HI-score.

Threshold values for haemodynamic variables were defined as follows: SAP of 160 mmHg or less, MAP at least 60 mmHg and HR at least 50 bpm and 100 bpm or less. We chose these values as they represent clinically accepted thresholds for triggering measures to restore stability. The association between the deviation of BP from accepted norms, mortality and other adverse post- operative events encouraged us to assign points according to the degree of deviation from these norms on an incremental basis (1,4,10–12).

Thresholds for all other variables were determined using the entire development cohort. Either quartiles or tertiles were determined for each variable and these were subsequently applied as threshold values in the validation cohort. Incremental points were assigned to each quartile or tertile. The time of a variable being outside each of the haemodynamic targets was determined. In view of the large interindividual variation in duration of the surgical procedure this was assessed as the percentage of intra-operative time and scored incrementally. Volume therapy was assessed as mean infusion rate per kilogram of body weight (ml kg^{-1} h^{-1}) of the cumulative amount of intra-operatively administered fluids, to correct for procedure duration and body weight. Mean infusion rates of 84 ml h^{-1} or less (our institutional standard baseline infusion rate) was considered normal. The mean infusion rate per kilogram of body weight of each administered vasoactive and inotropic drug was calculated to correct the cumulative dose for procedure duration and body weight. Included drugs were norepinephrine, phenylephrine and dobutamine. Since the cardiovascular potency of these drugs varies considerably, norepinephrine was assigned the most points followed by phenylephrine and dobutamine. Incremental scores per drug were assigned according to infusion rates.

The maximum score assigned to each of the three main domains was weighted at 40-30-90 points for haemodynamic variables, volume therapy and vasoactive medication, respectively. Weights were assigned based on consensus between the investigators. In view of the importance of interventions to maintain haemodynamic stability, volume therapy and administration of vasoactive medication combined were assigned a triple weight compared with haemodynamic variables (i.e. 120 vs. 40 points). Vasoactive medication use was assigned a triple weight relative to volume therapy because of its much stronger effect in increasing BP (14). Thus the range of the total HI-score varied from 0 to 160 points. A complete overview of all components including threshold values for corresponding scores is provided in Table 1.

Statistical analysis

A descriptive analysis was performed for all variables. Continuous variables are reported as mean (SD) or median [interquartile range] where appropriate and categorical data as counts and proportions. Missing data were not replaced. Means, medians and proportions were analysed using Student *t*, Mann–Whitney *U* and the χ^2 or Fisher exact tests as appropriate. Relationships between the HI-score and continuous or categorical variables were determined using Spearman correlation coefficients (r_s) or the Kruskal–Wallis test, as appropriate. Multivariable linear regression analyses were carried out to assess the relationship of the HI-score with group allocation (HRS or HI-score, haemodynamic instability score; HR, heart rate; MAP, mean arterial pressure; SAP, systolic arterial pressure. control) taking account of age, sex, BMI and American Society of Anesthesiologists (ASA) physical status. Two- sided *P* values less than 0.05 were considered statistically significant. Statistical Package for Social Sciences (SPSS) 23.0 for Windows (IBM SPSS Statistics, IBM Corporation, Armonk, New York, USA) was used for the statistical analysis. We adhered to the transparent reporting of a multivariable prediction model for individual prognosis or diagnosis (TRIPOD) statement (15).

Table 1: Threshold values for all haemodynamic instability score components

Domain	HI-score component	Value	Score
Haemodynamic variables	Maximum SAP (mmHg)	<160	0
		160 to 179	1
		180 to 199	3
		>200	7
	Time SAP > 160 mmHg (%)	0	0
		0.1 to 1.0	1
		1.1 to 6.6	3
		>6.7	7
	Minimum MAP (mmHg)	>60	0
		50 to 59	1
		40 to 49	3
		<40	7
	Time MAP < 60 mmHg (%)	0	0
		0.1 to 1.1	1
		1.2 to 4.1	3
		>4.2	7
	Maximum HR (bpm)	<100	0
		100 to 119	1
		>120	3
	Time HR >100 bpm (%)	0	0
		0.1 to 1.0	1
		>1.0	3
	Minimum HR (bpm)	>50	0
		40 to 49	1
		<40	3
	Time HR <50 bpm (%)	0	0
		0.1 to 1.7	1
		>1.7	3
Volume therapy	Volume therapy (ml kg^{-1} h^{-1})	0 to 84	0
		<6.3	2
		6.4 to 9.7	6
		9.8 to 14.3	14
		>14.3	30
Cardiovascular medication	Norepinephrine (mg kg^{-1} h^{-1})	0	0
		>0 to 1.48	5
		1.49 to 2.47	15
		2.48 to 4.14	35
		>4.14	75
	Phenylephrine (mg kg^{-1} h^{-1})	0	0
		>0.0 to 2.06	4
		>2.06	12
	Dobutamine (mg kg^{-1} h^{-1})	0	0
		>0 to 0.22	1
		>0.22	3
Total			0 to 160

Results

In total, 450 eligible patients were identified of whom 114 were excluded due to lack of intra-arterial BP measurement, four because of the administration of uncommon vasoactive or inotropic drugs and one due to incomplete data (Fig. 1), leaving 203 HRS patients and 128 controls for analysis. The development cohort was randomly selected and consisted of 50 controls and 50 HRS patients. The validation cohort consisted of the 231 remaining patients (153 HRS patients and 78 controls).

Figure 1: Flowchart of study subjects.

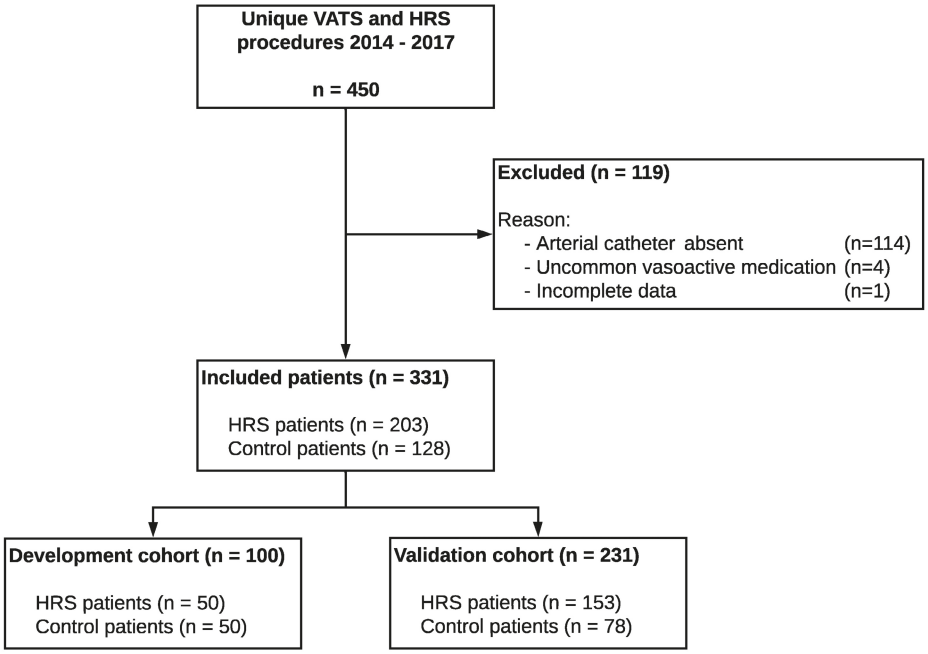


Table 2 shows the characteristics of the development cohort and the validation cohort, and also the separate HRS and control groups of both cohorts. All patients received total intravenous anaesthesia. Patients in the validation-control group were younger compared with the validation-HRS group [62 (11) vs. 66 (12) years, $P = 0.027$]. In the validation-control group, the ASA physical status class was significantly higher and the procedure time was shorter compared with the validation-HRS group, 138 [87 to 329] vs. 287 [175 to 440] min ($P < 0.001$). Age, sex, BMI, ASA physical status and procedure duration were similar in the development and validation cohorts (data not shown). All components off the HI-score are presented in Table 3.

Table 2: Patient characteristics

	Development			Validation			Control vs. HRS
Variables	**Total, *n*= 100**	**Control, *n*= 50**	**HRS, *n*= 50**	**Total, *n*= 231**	**Control, *n*= 78**	**HRS, *n*= 153**	***P* value**
Age (year)	62 (± 12)	58 (± 11)	66 (± 11)	65 (± 11)	62 (± 11)	66 (± 12)	0.027
Male sex	62 (62)	32 (64)	30 (60)	131 (57)	42 (54)	89 (58)	0.58
BMI (kg m^{-2})	26.9 (± 5.3)	27.8 (± 5.9)	26.0 (± 4.5)	26.6 (± 4.6)	27.2 (± 4.3)	26.3 (± 4.7)	0.18
ASA							0.007
Class I	4 (4)	1 (2)	3 (6)	12 (5)	2 (3)	10 (7)	
Class II	41 (41)	10 (20)	31 (62)	90 (39)	18 (23)	72 (47)	
Class III	43 (43)	27 (54)	16 (32)	108 (47)	40 (51)	68 (44)	
Class IV	3 (3)	3 (6)	0 (0)	8 (3)	5 (6)	3 (2)	
Unknown	9 (9)	9 (18)	0 (0)	13 (6)	13 (17)	0 (0)	
Procedure type							–
VATS	50 (50)	50 (100)		78 (34)	78 (100)		
HIPEC	6 (6)		6 (12)	15 (6)		15 (10)	
PPPD	3 (3)		3 (6)	22 (10)		22 (14)	
APR	18 (18)		18 (36)	30 (13)		30 (20)	
Open AAA repair	2 (2)		2 (4)	10 (4)		10 (7)	
Oesophageal resection	3 (3)		3 (6)	18 (8)		18 (12)	
Femoral popliteal repair	11 (3)		11 (22)	39 (17)		39 (25)	
Total hip arthroplasty	7 (7)		7 (14)	19 (8)		19 (12)	
Procedure duration (min)	214 [111 to 368]	124 [78 to 311]	303 [204 to 449]	239 [133 to 379]	138 [87 to 329]	287 [175 to 440]	<0.001

Data presented as mean (SD), median [IQR] or number (percentage). AAA, abdominal aortic aneurysm; APR, abdominal perineal resection; ASA, American Society of Anesthesiologists physical status score; HIPEC, hyperthermic intraperitoneal chemotherapy; HRS, high-risk abdominal surgery; PPPD, pylorus-preserving pancreati coduodenectomy; VATS, video-assisted thoracoscopic surgery.

Table 3: Haemodynamic instability score components

Variables	Development, *n*= 100	Validation			Control vs. HRS *P* value
		Total, *n*= 231	Control, *n*= 78	HRS, *n*= 153	
Haemodynamics					
Maximum SAP (mmHg)	162 [145 to 182]	160 [146 to 177]	151 [141 to 165]	165 [149 to 182]	<0.001
Duration SAP > 160 mmHg (%)	0.1 [0.0 to 2.1]	0.0 [0.0 to 1.0]	0.0 [0.0 to 0.1]	0.2 [0.0 to 2.1]	<0.001
Minimum MAP (mmHg)	53 [44 to 58]	53 [47 to 58]	53 [48 to 60]	53 [46 to 58]	0.47
Duration MAP < 60 mmHg (%)	1.4 [0.2 to 4.8]	1.7 [0.1 to 5.2]	2.3 [0.0 to 5.7]	1.5 [0.3 to 4.6]	0.71
Maximum HR (bpm)	103 [87 to 121]	103 [89 to 120]	114 [99 to 136]	99 [88 to 113]	<0.001
Duration HR > 100 bpm (%)	0.1 [0.0 to 1.1]	0.1 [0.0 to 2.1]	0.3 [0.0 to 9.1]	0.0 [0.0 to 1.1]	<0.001
Minimum HR (bpm)	47 [40 to 53]	49 [41 to 59]	51 [38 to 63]	48 [42 to 59]	0.88
Duration HR < 50 bpm (%)	0.2 [0.0 to 3.6]	0.0 [0.0 to 3.5]	0.0 [0.0 to 1.9]	0.2 [0.0 to 4.9]	0.10
Volume therapy					
Infusion rate (ml h^{-1})	804 [573 to 1076]	846 [631 to 1056]	676 [508 to 1003]	881 [683 to 1078]	0.001
Infusion rate (ml kg^{-1} h^{-1})	9.7 [6.25 to 14.26]	10.6 [7.8 to 14.6]	8.7 [5.9 to 14.5]	11.3 [9.1 to 14.7]	0.001
Medication					
Norepinephrine	77 (77)	199 (86)	54 (69)	145 (95)	<0.001
Norepinephrine (mg kg^{-1} h^{-1})	2.47 [1.48 to 4.14]	2.86 [1.61 to 5.18]	2.99 [1.46 to 3.99]	2.85 [1.70 to 5.72]	0.12
Phenylephrine	7 (7)	21 (9)	9 (12)	12 (8)	0.47
Phenylephrine (mg kg^{-1} h^{-1})	2.06 [0.36 to 11.70]	9.31 [3.23 to 14.99]	12.74 [4.79 to 22.13]	5.64 [2.82 to 13.06]	0.19
Dobutamine	5 (5)	9 (4)	0 (0)	9 (6)	0.030
Dobutamine (mg kg^{-1} h^{-1})	0.22 [0.17 to 0.50]	0.11 [0.04 to 0.17]	–	0.11 [0.04 to 0.17]	–

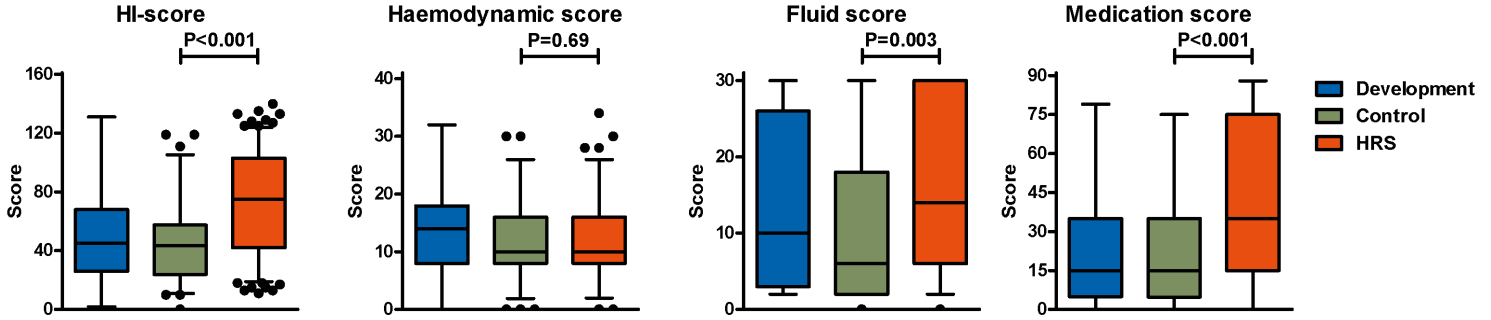
Data are presented as median [IQR] or number (percentage). HR, heart rate; HRS, high-risk abdominal surgery; MAP, mean arterial pressure; SAP, systolic arterial pressure.

The total HI-score was 45 [26 to 68] in the development cohort. The haemodynamic, volume therapy and vasoactive medication components were 14 [8 to 18], 10 [3 to 26], 15 [5 to 35], respectively, in the development cohort.

The total HI-score in the validation cohort was higher in the HRS group compared with the control group, 59 [37 to 96] vs. 44 [24 to 58], ($P < 0.001$, Fig. 2). The haemodynamic component of the HI-score was not significantly different between the control and HRS group of the validation cohort, 10 [8 to 16] vs. 10 [8 to 16], (P 0.69, Fig. 2). However, the HI-score components for volume therapy and vasoactive medication in the validation cohort were both higher in the HRS group compared with the control group, 14 [6 to 30] vs. 6 [2 to 18] (P 0.003) and 35 [15 to 75] vs. 15 [5 to 35] ($P < 0.001$, Fig. 2).

In the total validation cohort, the HI-score was negatively associated with BMI (r_s 0.27, $P < 0.001$) but not with age or sex (r_s 0.02, P 0.75 and r_s 0.05, P 0.49, respectively). The HI-score was significantly higher for ASA physical status 4 at 79 [56 to 123] compared with 63 [48 to 99], 54 [35 to 91], 49 [29 to 79] and for ASA physical status 1, 2 and 3 respectively, (P 0.03). Multivariable linear regression analysis was subsequently carried out to determine the independent relationship of the HI-score with group allocation (HRS or control) taking into account age, sex, BMI and ASA physical status. HI-score was independently and positively related to HRS (β= 0.30, 95% CI: 0.18 to 0.46, $P < 0.001$) and ASA physical status class 4 (β= 0.17 95% CI: 0.01 to 0.33, P= 0.046), while a negative relationship was found with BMI (β= -0.25, 95% CI: -0.38 to -0.12, $P < 0.001$).

Figure 2: Boxplots demonstrating the haemodynamic instability score and its separate domains in the development cohort and the control and high-risk abdominal surgery groups of the validation cohort. Boxes represent median and IQR, whiskers represent 5th and 95th percentile and dots represent outliers.



Discussion

We present the development and validation of a novel comprehensive scoring method that rates the degree of intra-operative haemodynamic instability. The HI-score was significantly higher in a high-risk surgery group compared with a low-risk. It has proved a useful descriptive tool for quantifying the degree of haemodynamic instability associated with surgical procedures of different risk categories independent from baseline differences between these two groups.

There is no consensus on how to define haemodynamic instability and the lack of a generally accepted reference standard for comparison hampers validation of any scoring system intended to quantify haemodynamic instability. Our approach was to determine normalised threshold values for all components of the HI-score in a development cohort of surgical patients that could be expected to represent a wide range of the haemodynamic instability spectrum. Previously only a few scoring systems have been developed to assess overall haemodynamic instability,(16,17) but a common flaw of these systems is that they only contain haemodynamic variables, without adjustment for stabilising therapy. Since deviation of haemodynamic variables together with the amount of administered vasoactive agents are associated with morbidity and mortality it seems logical to take these factors into account when determining the degree of haemodynamic instability (1–5,7,8). A disregard for stabilising therapies is a potential confounding factor. Support for this can be found in the failure to see a difference in the haemodynamic component of the HI-score between the two groups despite an evident difference in corrective measures. The vasoactive medication score and volume therapy score were significantly higher in the HRS group. Of note, studies that have previously demonstrated an association between haemodynamic instability and mortality were also not corrected for stabilising measures, which further underscores the potential importance of the HI- score (4).

The HI-score might be particularly valuable in a research setting where there is a need to determine haemodynamic instability as a continuous single variable outcome. The HI-score can be easily applied since the different components of the score are usually available. In addition to comparing different types of surgery, the HI-score can also be applied to compare different interventions during the same type of surgery, to identify predictors of haemodynamic instability or to quantify the effect of interventions that aim to prevent or correct a certain degree of haemodynamic instability. Furthermore, its application might be extended to intensive care unit (ICU) cohorts where similar methodological problems with

respect to the assessment of haemodynamic instability occur. A clinical application of the HI-score might be to assist postoperative triage of patients either to the ICU or regular postoperative care unit, or to identify those patients who are at increased risk for postoperative complications. However, prospective confirmation studies are warranted for proper external validation and to determine the relationship of the HI-score with clinical endpoints such as morbidity and mortality.

Major strengths of the current study are the well defined cohorts, derivation of high-quality data from the PDMS and relatively easy application of the HI-score in future research. Because the type of vasoactive medication administered to a target group might differ from our development cohort, for future studies it might be necessary to redistribute the 90 points for vasoactive medication while taking different cardiovascular potencies into account.

The retrospective design of the study can be considered a limitation of our study. Haemodynamic targets during the procedure were not standardised and interventions by using either volume therapy or vasoactive medication were at the discretion of the anaesthesiologist and reflect common practice. The HI-score is, by design, able to adjust for differences in management as it incorporates many relevant determinants into a single score. The use of threshold values for haemodynamic variables can also be considered a weakness of the current study. In our opinion, however, the chosen threshold values reflect common clinical practice. After correction for differences in possible patient related risk factors, we found that HRS-surgery was still independently associated with a higher HI-score. Incorporation of the anaesthetic induction period into the HI-score may also be of relevance for future studies (18).

In conclusion, we developed and internally validated a novel and comprehensive scoring system to grade intra operative haemodynamic instability by combining haemodynamic variables and treatment measure that aim to improve haemodynamic stability. We demonstrated that the HI-score was significantly different between surgical procedures associated with low or high degrees of haemodynamic instability. The HI-score provides a clinical tool that may have applications in both patient management and clinical research.

Acknowledgements

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Chapter 9

Efficacy of phenoxybenzamine versus doxazosin on hemodynamic control during pheochromocytoma resection - a randomized controlled trial

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Abstract

Background: Pretreatment with α-adrenergic receptor blockers is recommended to prevent catecholamine induced hemodynamic instability during resection of a pheochromocytoma or a sympathetic paraganglioma (PPGL).

Objective: To determine which type of α-adrenergic receptor blocker provides the best efficacy.

Design: multicenter randomized controlled open-label trial.

Setting: 9 centers in The Netherlands.

Patients: 134 patients with non-metastatic PPGL.

Interventions: Randomized treatment with phenoxybenzamine or doxazosin starting 2-3 weeks before surgery using a blood pressure targeted titration schedule. Intraoperative hemodynamic management was standardized.

Measurements: The primary efficacy endpoint was the cumulative intraoperative time outside the blood pressure target range (i.e., SBP >160 mmHg or MAP <60 mmHg) expressed as a percentage of total surgical procedure time. A validated hemodynamic instability score was designated as a secondary efficacy outcome.

Results: The median cumulative time outside blood pressure targets was 11.1% [IQR: 4.3-20.6] in the phenoxybenzamine group compared to 12.2% [5.3-20.2] in the doxazosin group (P=0.75, *r*=0.03). Intraoperative hemodynamic instability quantified by the hemodynamic instability score was 38.0 [28.8-58.0] and 50.0 [35.3-63.8] in the phenoxybenzamine and doxazosin group, respectively (P=0.020, *r*=0.20). The 30-day cardiovascular complication rate was 8.8% and 6.9% in the phenoxybenzamine and doxazosin group, respectively (P=0.68). There was no mortality after 30 days.

Limitations: study medication was not blinded for safety reasons. Not all participants reached the preoperative blood pressure targets.

Conclusion: The duration of blood pressure outside the target range during resection

of a PPGL was not different after preoperative treatment with either phenoxybenzamine or doxazosin. However, phenoxybenzamine was more effective in preventing intraoperative hemodynamic instability, suggesting that phenoxybenzamine might be preferred for preoperative treatment of patients before resection of a PPGL.

Registration: Clinicaltrials.gov NCT01379898.

Funding Source: Unrestricted grant from Ipsen pharmaceuticals

Introduction

Pheochromocytoma and sympathetic paraganglioma (PPGL) are neuro-endocrine tumors originating from chromaffin cells in the adrenal medulla and extra-adrenal sympathetic paraganglia, respectively (1). Overproduction of catecholamines is a key feature of PPGL and responsible for an increased cardiovascular risk (2-4). Curative surgical resection is the treatment of choice except in cases of metastatic disease (5).

Resection of a PPGL is associated with a high risk of hemodynamic instability and subsequent cardiovascular complications due to uncontrolled release of catecholamines in response to various anesthesiological and surgical stimuli (6-8). In order to minimize intraoperative hemodynamic instability, pretreatment with an α-adrenergic receptor blocker is recommended to antagonize the α-receptor mediated vasoconstrictive effects of catecholamines (5, 9). Two frequently prescribed drugs for this purpose are phenoxybenzamine, a nonselective and non- competitive α_1- and α_2-adrenergic receptor blocker, and doxazosin, a selective and competitive α_1-adrenergic receptor blocker. Studies evaluating pretreatment with either phenoxybenzamine or doxazosin have shown conflicting results with respect to intraoperative blood pressure levels outside of a certain target range (10-13). Without exception these studies were non-randomized and retrospective in design. Apart from blood pressure levels, hemodynamic instability is also reflected by the amount of vasoactive medication and intravenous fluids required to correct an abnormal blood pressure (14-16). The present randomized multicenter study was initiated to compare the efficacy of pretreatment with either phenoxybenzamine or doxazosin on the intraoperative hemodynamic stability during PPGL resection.

Methods

Trial Design

Pheochromocytoma Randomized Study Comparing Adrenoreceptor Inhibiting agents for Preoperative Treatment (PRESCRIPT) was an investigator-initiated multicenter, randomized controlled, open-label trial conducted between January 2012 and December 2017 at nine sites in The Netherlands. The trial protocol was approved by the institutional review board of the University Medical Center Groningen, University of Groningen, The Netherlands, in compliance with the Dutch Medical Research Involving Human Subjects Act and the Declaration of Helsinki. All patients provided written informed consent. The PRESCRIPT trial has been registered under ClinicalTrials.gov number NCT01379898. The protocol and

statistical analysis plan are provided in the appendix. The Consolidated Standards of Reporting Trials statement was followed for presentation of the current study (17).

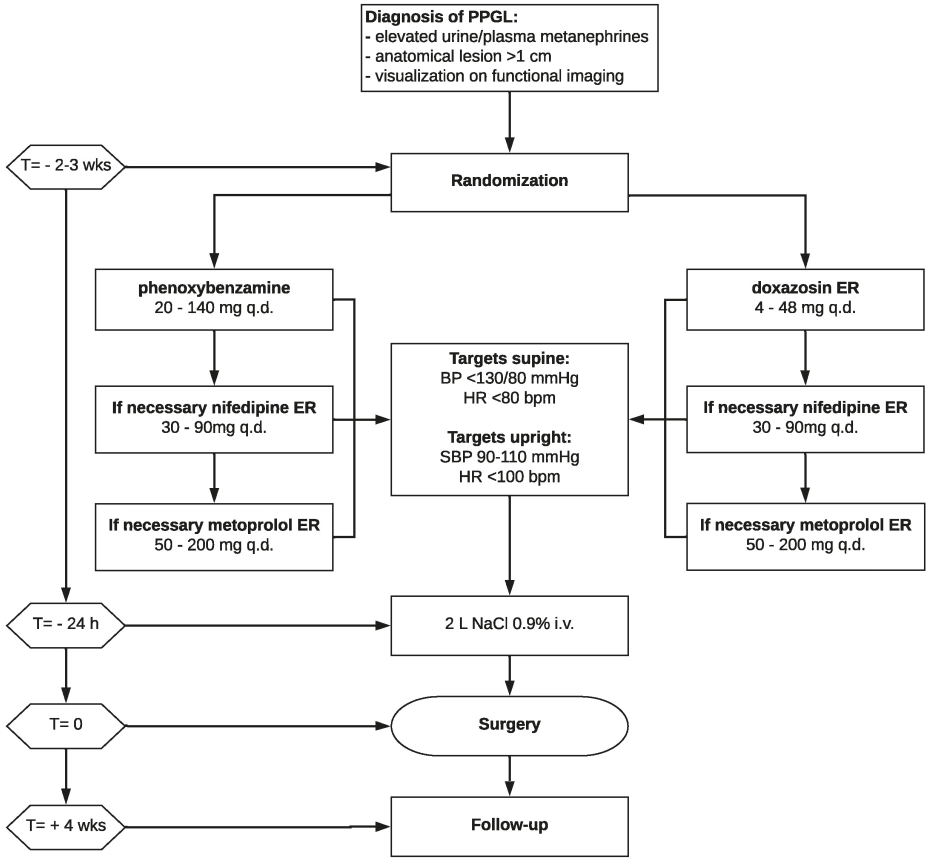
Participants

Adult patients aged 18 years or older with a recently diagnosed PPGL and an indication for surgical resection were considered eligible. Inclusion criteria were a diagnosis of non-metastatic PPGL with elevated plasma or urinary (nor)metanephrine concentrations, a minimum tumor diameter of 1 cm on CT or MRI, and visualization on functional imaging (e.g. I^{123}-MIBG scintigraphy or [^{18}F]DOPA-PET). Exclusion criteria were metastatic PPGL, severe hemodynamic instability necessitating presurgical admission to the intensive care unit, or pregnancy.

Randomization and Procedures

Patients were randomized to pretreatment with either phenoxybenzamine or doxazosin extended-release in a 1:1 ratio using randomly permuted blocks with alternating block sizes of two and four stratified by center with interactive web-based randomization software. Before the start of pretreatment, blood samples were drawn after 30 minutes of supine rest and stored at - 80°C until determination of plasma free (nor)metanephrine and catecholamines concentrations using high-pressure liquid chromatography tandem mass spectrometry (LC-MS/MS) with online solid-phase extraction in a central reference laboratory (18). Treatment was started 2-3 weeks before surgery using blood pressure guided dose titration with a maximum dosage of 70 mg phenoxybenzamine twice daily or 24 mg doxazosin twice daily (Figure 1), in accordance with the maximum dosages previously reported for this indication (10). It was at the discretion of the treating physician whether the drug treatment would take place in the outpatient or inpatient clinic. During the whole pretreatment period, blood pressure and heart rate were measured twice daily with a certified automated electronic blood pressure monitor just before ingestion of the study drugs. Each measurement consisted of a single recording after 5 minutes of supine rest and subsequently after 3 minutes in upright posture. Blood pressure and heart rate measurements were either performed at home by the patients themselves after careful instructions, or at the hospital by medical personnel. Target values were a blood pressure <130/80 mmHg in the supine position and a systolic blood pressure between 90-110 mmHg in the upright position (19). Nifedipine extended-release 30-90 mg once daily was added when these targets were not reached despite a maximum dosage of either study drug. Heart rate target values were <80 bpm and <100 bpm in the supine and upright position, respectively.

Figure 1: Flow-chart of the trial procedure.



BP: blood pressure, HR: heart rate, ER: extended-release, i.v.: intravenous.

Metoprolol extended-release 50-200 mg once daily was added in case these targets were not achieved. In addition, patients were advised to consume a diet containing at least 15 grams of sodium chloride per day (5). During the last 24 hours before surgery, two liters of 0.9% saline was administered intravenously. Resection of the PPGL was postponed if the supine blood pressure was >160/100 mmHg on the day before surgery. In each participating center, patients were treated by a dedicated team of endocrinologists, surgeons, and anesthesiologists.

Blood pressure and heart rate during surgery were monitored by continuous intra-arterial measurement. Hemodynamic management was performed using a standardized operating procedure describing in detail the anesthesiological

procedures including the indications for pharmacological interventions and the preferred vasoactive medication (Appendix Table 1). Intraoperative hemodynamic targets were: systolic blood pressure <160 mmHg, mean arterial pressure >60 mmHg, and heart rate <100 bpm. Administration of vasoactive medication was only allowed when hemodynamic variables were outside these targets. After surgery, patients were monitored at the post-anesthesia or intensive care unit. Postoperative pharmacological interventions to correct hemodynamic deviations were applied according to the standard operating procedure. We extracted all data on blood pressure, heart rate, intravenous volume therapy, and vasoactive medication from the electronic patient data monitoring system starting at induction of anesthesia and ending at discharge from the post-anesthesia care unit or intensive care unit. Both duration and amplitude of hemodynamic variables outside the target range were assessed and cumulative dosages of vasoactive medication were calculated.

Outcome Measures

The primary endpoint of our study was the cumulative intraoperative time outside the blood pressure target range, expressed as a percentage of the time interval between induction of anesthesia (i.e. first administration of propofol) and suturing of the incision. As a secondary efficacy endpoint, we used the Hemodynamic Instability score (HI-score), a validated semi- quantitative score reflecting the degree of hemodynamic instability (16). In short, the HI-score consists of three components: hemodynamic variables (i.e., blood pressure and heart rate), cumulative dosage of vasoactive medication, and fluid therapy. For each of these three components, incremental points are attributed according to the magnitude of deviation from predefined thresholds as well as infusion rates of vasoactive drugs and fluids. Thus, a higher HI- score represents a higher degree of overall hemodynamic instability. For the present study, we modified the original HI-score by including the dosages of vasodilating drugs and b-adrenergic receptor blockers (Appendix Table 2).

Other secondary efficacy endpoints were i) the frequency, duration, and magnitude of a systolic blood pressure >160 mmHg, mean arterial pressure <60 mmHg, and heart rate >100 bpm; ii) number and cumulative dosages of intra-operatively administered vasoactive drugs; and iii) duration of postoperative administration of vasopressive drugs. Safety endpoints were cardiovascular complications and mortality from the first administration of study medication until 30 days after surgery. In addition, the frequency of postoperative glucose levels ≤3.5 mmol/L and length of hospital stay were assessed. Preoperative adverse events were assessed and graded according to the Common Terminology Criteria for Adverse Events (20).

Statistical Analysis

The sample size was calculated at a total of 134 subjects in order to demonstrate a relative reduction of 20% in intraoperative time outside the predefined blood pressure targets, assuming a frequency of 8 ± 4%, between patients pretreated with phenoxybenzamine or doxazosin with a power of at least 80% and a two-sided alpha of 0.05. Patients who never received the allocated treatment were excluded from all analyses. We performed all efficacy and exploratory analyses in a modified intention-to-treat population, meaning that we excluded subjects in whom pathological examination of the resected tumor was inconsistent with a PPGL since these patients were not at risk for catecholamine induced hemodynamic instability. The safety analysis was performed in all patients who received the allocated treatment, including the cases in which another pathological diagnosis than PPGL was established (Appendix Figure 1). Continuous variables are presented as mean ± SD or median [IQR] where appropriate. Categorical variables are presented as absolute number or percentages. Continuous variables were compared using a t-test or Mann-Whitney U test. Non-parametrical effect sizes were calculated using Rosenthals' formula (21). Categorical data were analyzed using Chi-square or Fisher's exact test. Two-sided P-values <0.05 were considered significant. All statistical analyses were carried out with SPSS version 23 (IBM Corporation, Armonk, NY, USA).

Exploratory Analyses

Exploratory analyses were carried out in order to assess the relationship between efficacy endpoints and cardiovascular complications. In addition, determinants of hemodynamic instability were explored for identification of potential risk factors. The relationship between achievement of preoperative blood pressure targets and intraoperative hemodynamic instability was assessed in a multivariable regression model. Further details are provided in the appendix.

Role of the Funding Source

This trial was supported by an unrestricted grant from the Ipsen pharmaceutical company. The funder of the study had no role in study design, data collection, data analysis, data interpretation, or in writing of the report. The authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Participants

A total of 144 patients were enrolled in the trial. Four patients were excluded from all analyses because the allocated treatment was never initiated, leaving 140 patients who completed the study. Notably, in six patients the final pathology report did not reveal a PPGL (Appendix Figure 1). Thus, a total of 134 patients met the criteria for the modified intention-to-treat population (phenoxybenzamine group: n=66, doxazosin group: n=68). The safety analysis was performed using the data of all 140 patients who completed the study (Appendix Figure 1).

Baseline characteristics and preoperative blood pressure values are presented in Table 1. There were no differences between the two groups with respect to demographic characteristics, cardiovascular risk factors, ASA physical score, plasma free (nor)metanephrine, or catecholamine secretion patterns. The median duration of pretreatment was 14 days in both groups, and patients received a median dosage of 120 [78-140] mg phenoxybenzamine or 40 [32-48] mg doxazosin on the day before surgery. A calcium channel blocker was administered to 42.4% of the patients in the phenoxybenzamine group compared to 39.7% in the doxazosin group (P=0.86). A higher proportion of patients in the phenoxybenzamine group received metoprolol (89.4% vs. 66.2%, P=0.002), which was also prescribed at higher dosages.

Efficacy Outcomes

The primary endpoint, i.e. the median cumulative time outside the blood pressure target range during surgery, was 11.1% [4.3-20.6] in the phenoxybenzamine group compared to 12.2% [5.3- 20.2] in the doxazosin group (P=0.75, *r*=0.03, Figure 2). The median total HI-score was lower in the phenoxybenzamine group compared to the doxazosin group (38.0 [28.8-58.0] vs. 50.0 [35.3- 63.8], P=0.02, *r=0.20*). Peak systolic blood pressure, cumulative time and frequency of systolic blood pressure >160 mmHg, and the amount of vasodilating drugs were all lower in the phenoxybenzamine group (Table 2). Frequency and duration of a mean arterial pressure <60 mmHg or heart rate >100 bpm were not different between groups (Table 2). There were no differences between phenoxybenzamine and doxazosin with respect to the occurrence of postoperative hypotension (40.0% vs. 38.8%, P>0.99), the proportion of patients requiring vasopressors (33.3% and 32.4%, P>0.99), or the duration of vasopressor treatment (402 [161-1185] vs. 490 [163-1167] min, P=0.98).

Table 1: Patient characteristics

Characteristic		Phenoxybenzamine (n=66)	Doxazosin (n=68)	
Female		34 (51.5%)	36 (52.9%)	
Age (years)		54 ± 15	54 ± 15	
BMI (kg/m2)		25.6 [23.6-29.0]	25.1 [22.4-29.1]	
Smoking				
never		28 (42.4%)	32 (47.1%)	
previous		18 (27.3%)	19 (27.9%)	
current		20 (30.3%)	17 (25.0%)	
Prior cardiovascular event [a]		17 (25.8%)	11 (16.7%)	
ASA class				
I		11 (16.7%)	10 (14.7%)	
II		34 (51.5%)	43 (63.2%)	
III		20 (30.3%)	15 (22.1%)	
IV		1 (1.5%)	0 (0%)	
Germline mutation				
yes		17 (25.8%)	17 (25.0%)	
no		37 (56.1%)	37 (54.4%)	
not assessed		12 (18.2%)	14 (20.6%)	
Tumor localization				
unilateral pheochromocytoma		59 (89.4%)	65 (95.6%)	
bilateral pheochromocytoma		5 (7.6%)	1 (1.5%)	
sympathetic paraganglioma		2 (3.0%)	2 (2.9%)	
Maximum tumor diameter (mm)		38 [28-51]	42 [29-61]	
Biochemical profile				
plasma free metanephrine (nmol/L)		1.37 [0.29-5.64]	1.04 [0.21-3.39]	
plasma free normetanephrine (nmol/L)		4.33 [1.63-10.11]	3.41 [1.52-8.44]	
plasma epinephrine (nmol/L)		0.48 [0.23-2.13]	0.40 [0.19-1.41]	
plasma norepinephrine (nmol/L)		4.47 [2.91-11.91]	4.87 [3.03-17.69]	
Duration of pretreatment (days)		14 [13-20]	14 [13-19]	
Medication on day before surgery				
daily dosage study drug (mg)		120 [78-140]	40 [32-48]	
patients receiving any CCB		28 (42.4%)	27 (39.7%)	
daily dosage nifedipine (mg)[b]		60 [30-90]	60 [30-90]	
patients receiving any β-blocker		59 (89.4%)	45 (66.2%)	f
daily dosage metoprolol (mg)[c]		100 [50-150]	50 [50-100]	g
Hemodynamic variables at randomization				
Supine	SBP (mmHg)	144 [124-156]	138 [122-152]	
	DBP (mmHg)	82 [73-88]	80 [72-87]	
	HR (bpm)	76 [66-85]	71 [63-78]	h
Upright	SBP (mmHg)	136 [122-151]	138 [124-151]	
	DBP (mmHg)	84 [78-94]	85 [77-93]	
	HR (bpm)	87 [73-98]	82 [76-94]	
Hemodynamic variables day before surgery				
Supine	SBP (mmHg)	132 [116-143]	124 [115-138]	
	DBP (mmHg)	74 [67-84]	69 [63-80]	i

Table 1: Continued

Characteristic		Phenoxybenzamine (n=66)	Doxazosin (n=68)	
	HR (bpm)	73 [64-83]	71 [65-80]	
Upright	SBP (mmHg)	120 [107-133]	120 [104-130]	
	DBP (mmHg)	71 [65-81]	71 [64-82]	
	HR (bpm)	90 [83-106]	86 [74-98]	j
Preoperative targets achieved				k
supine BP <130/80 + upright SBP 90-110		16 (24.6%)	13 (19.7%)	
supine BP <130/80		13 (20.0%)	28 (42.4%)	
upright SBP 90-110		1 (1.5%)	5 (7.6%)	
none		35 (53.8%)	20 (30.3%)	
Surgical approach				
laparoscopy		48 (72.7%)	44 (64.7%)	
laparotomy		9 (13.6%)	12 (17.6%)	
posterior retroperitoneoscopic		9 (13.6%)	12 (17.6%)	
Type of anesthesia				
total intravenous		40 (60.6%)	43 (63.2%)	
balanced inhalation		26 (39.4%)	25 (36.8%)	
Epidural anesthesia		7 (10.6%)	9 (13.6%)	
Anesthesia duration (min)[d]		140 [112-164]	145 [110-164]	
Surgical duration (min)[e]		95 [71-127]	99 [72-120]	

Data are represented as n (%), mean ± SD, or median [IQR].

BMI: body mass index, ASA: American Society of Anesthesiologists, CCB: calcium channel blocker, BP: blood pressure, SBP: systolic blood pressure, DBP: diastolic blood pressure

[a] History of coronary artery disease, heart failure, stroke, peripheral artery disease, or aortic aneurysm. [b] Nifedipine was prescribed in 87% of patients receiving any CCB. Median [IQR] shown of only these cases. In the remaining cases, amlodipine, barnidipine, or verapamil was prescribed. [c] metoprolol was prescribed in 88% of patients receiving any β-blocker. Median [IQR] shown of only these cases. In the remaining cases, propranolol, atenolol, or bisoprolol was prescribed. [d] Time from induction of anesthesia until suturing of the incision. [e] Time from incision until suturing of the incision. [f] P=0.002. [g] P=0.007. [h] P=0.02. [i] P=0.02. [j] P=0.03. [k] P=0.005.

Table 2: Secondary efficacy endpoints

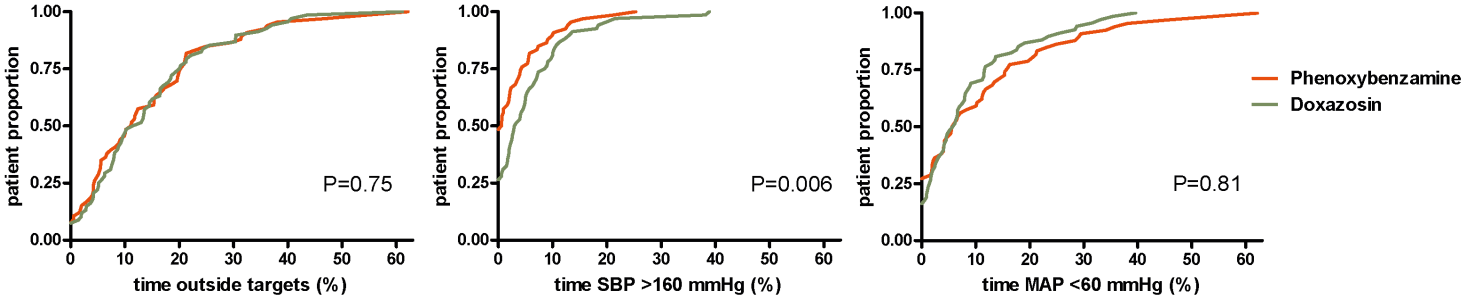
	Phenoxybenzamine (n=66)	Doxazosin (n=68)	P-value
Systolic blood pressure >160 mmHg			
frequency	34 (51.5%)	49 (72.1%)	0.02
duration (%)	0.6 [0.0-4.6]	3.1 [0.0-8.9]	0.005
Maximum SBP (mmHg)	163 [146-188]	181 [159-203]	0.005
Vasodilating drugs			0.02
0	29 (43.9%)	14 (20.6%)	
1	21 (31.8%)	23 (33.8%)	
2	10 (15.2%)	22 (32.4%)	
3	6 (9.1%)	8 (11.7%)	
4	0 (0%)	1 (1.5%)	
Cumulative dosage MgSO4 (g)	0 [0-3]	3 [0-4]	0.005
Cumulative dosage phentolamine (mg)	0 [0-0.5]	0 [0-4]	0.16
Mean arterial pressure <60 mmHg			
frequency	48 (72.7%)	56 (82.4%)	0.22
duration (%)	5.8 [0.0-16.0]	6 [1-12]	0.82
Minimum MAP (mmHg)	53 [44-60]	51 [46-57]	0.36
Vasoconstrictive/inotropic drugs			0.46
0	17 (25.8%)	13 (19.1%)	
1	24 (36.4%)	27 (39.7%)	
2	23 (34.8%)	22 (32.4%)	
3	2 (3.0%)	6 (8.8%)	
Infusion rate of fluids (ml/h)	632 [424-945]	636 [484-896]	0.81
Cumulative dosage phenylephrine (µg)	0 [0-425]	0 [0-300]	0.98
Cumulative dosage norepinephrine (µg)	55 [0-660]	139 [0-603]	0.52
Heart rate >100 bpm			
frequency	26 (39.4%)	33 (48.5%)	0.30
duration (%)	0.0 [0.0-2.4]	0.0 [0.0-3.2]	0.47
Maximum HR (bpm)	97 [85-115]	100 [85-115]	0.90
Esmolol (mg)	0 [0-0]	0 [0-0]	0.61

Data are presented as n (%) or median [interquartile range].

Adverse Events

There was no 30-day perioperative mortality in either treatment group. Perioperative complications are shown in Table 3. In each treatment group there were six cardiovascular complications, occurring in six patients of the phenoxybenzamine group and five patients of the doxazosin group (8.8% vs 6.9%, P=0.68). The number of subjects with postoperative hypoglycemia was not different (P=0.19). During pretreatment, adverse events were reported by 80.9% and 92.4% of the phenoxybenzamine and doxazosin users, respectively (P=0.08). All adverse events were graded as mild or moderate (i.e. grade I or II) and are listed in Appendix Table 3. The total length of hospital stay was 14 [7-19] and 14 [8-18] days in the phenoxybenzamine and doxazosin group, respectively (P=0.90).

Figure 2. Cumulative distribution of the percentage of total intraoperative time with blood pressure outside the target values, i.e. systolic blood pressure >160 mmHg and MAP <60 mmHg.



The X-axis represents the cumulative time outside of the respective blood pressure targets. The y-axis represents the cumulative proportion of patients.

Table 3: Perioperative complications

	Phenoxybenzamine (*n*=68)	**Doxazosin (*n*=72)**
Cardiovascular events		
asystole	0	1
atrial fibrillation/flutter	2	0
acute heart failure	3	2
pulmonary embolism	1	0
postoperative bleeding	0	2
intestinal necrosis	0	1
Infection		
pneumonia	4	5
urinary tract	2	1
wound	1	1
fever of unknown origin	0	1
Other		
excessive postoperative pain	1	1
delirium	1	0
intestinal perforation	0	1
hypoglycemia [a]	8	4

Data are presented as number of events.
[a] Glucose ≤3.5 mmol/L during the first 24 h postoperatively.

Exploratory Analyses

The primary endpoint in patients with (n=11) or without (n=123) a cardiovascular complication was 11.8% [4.9-33.0] and 11.3% [5.0-20.0], respectively (P=0.26). The associated HI-scores were 59.0 [43.8-73.0] and 42.5 [29.3-59.0], respectively (P=0.03). In patients with (n=104) or without (n=30) preoperative use of a β-adrenergic receptor blocker, the primary endpoint was 11.4% [5.2-21.0] and 10.8% [2.5-17.4], respectively (P=0.32). In addition, the associated HI-scores were 43.5 [32.3-59.0] and 49.0 [23.8-59.8] (P=0.84), respectively.

Univariate analysis demonstrated that tumor size, total plasma free metanephrines, and total plasma catecholamines were positively associated with the primary endpoint. Use of doxazosin, tumor size, total plasma free metanephrines, and total plasma catecholamines were positively associated with the HI-score (Appendix Table 4). These variables were subsequently tested in the multivariable linear regression model with the HI-score as a dependent variable. Total plasma free metanephrines did not contribute significantly to the model and was removed.

Achievement of different blood pressure targets was added. The final

model demonstrated that the use of doxazosin, tumor size, and total plasma catecholamines were positively associated with the HI-score (Appendix Table 5). The total model accounted for only a minority of the variance in HI-score (adjusted R2 0.16). Achievement of a supine blood pressure <130/80 mmHg, irrespective of the upright blood pressure, was negatively associated with the HI-score. Upright systolic blood pressure <90 mmHg was independently associated with an increased HI- score (Appendix Table 5).

Discussion

In this first randomized controlled trial in patients scheduled for resection of a PPGL we demonstrated that the cumulative time of blood pressure values outside the target range during PPGL surgery was not different after pretreatment with either phenoxybenzamine or doxazosin. Phenoxybenzamine was, however, more effective in preventing intraoperative systolic blood pressure above the target range and hemodynamic instability.

Treatment with an α-adrenergic receptor blocker prior to resection of a PPGL was first introduced in 1949 and has become part of routine clinical care since (22, 23). All previous studies on the type of α-adrenergic receptor blocker were retrospective in design and suffered from several biases, such as the use of historical controls and the lack of a well-defined perioperative management protocol (10-13). In addition, these studies applied different blood pressure targets during surgery and raised conflicting results (10-13).

It should be noted that comparable intraoperative blood pressure levels can be achieved with the administration of a variable amount of vasoactive drugs and intravenous fluids by the anesthesiologist. The extent of these interventions has been acknowledged as a fundamental marker of hemodynamic instability (14-16). Therefore, we have recently developed and validated a clinical score for assessment of hemodynamic instability during surgery (16). Using this score as a secondary endpoint, we found a lesser degree of intraoperative hemodynamic instability after pretreatment with phenoxybenzamine. In particular, patients in the phenoxybenzamine group demonstrated a shorter duration of systolic blood pressure above 160 mmHg, a lower peak systolic blood pressure, and a concomitant lower requirement of vasodilating drugs. This suggests that phenoxybenzamine offers a more effective inhibition of the α-adrenergic receptor than doxazosin, which might be explained by its non-competitive antagonism

compared to the competitive binding provided by doxazosin. Pretreatment with phenoxybenzamine did not result in more severe or a longer duration of postoperative hypotension, as previously suggested (24). We assume that this risk was minimized by the concomitant use of a high-sodium diet and the intravenous administration of saline the day before surgery (19). The higher rate of co- administration of β-adrenergic receptor blockers among patients allocated to phenoxybenzamine can be explained by the occurrence of reflex tachycardia as a result of inhibition of the presynaptic α_2-adrenergic receptor. Of notice, neither the primary endpoint nor the hemodynamic instability score was affected by preoperative use of β-adrenergic receptor blockers.

The relevance of a more stable hemodynamic profile is underlined by the observation that patients who developed a postoperative cardiovascular complication had a higher hemodynamic instability score, despite the absence of a difference in primary endpoint. This observation is in agreement with other studies describing the adverse effects of hemodynamic instability on postoperative outcome (9, 14, 15, 25-29). The rate of cardiovascular complications was not different between the treatment groups, but it should be noted that our study was not powered for this endpoint. In view of the rarity of PPGL and the current complication rate, it would not be feasible to enroll the number of patients required to demonstrate a relevant difference in perioperative cardiovascular events (30). The absence of mortality in our study is in agreement with the literature (9, 31, 32). In the past decades, the perioperative mortality has decreased dramatically, most likely as a result of improvement of the medical management with use of α-adrenergic receptor blockers and major technical advances in both anesthesiology and surgery (7, 19, 33).

The major strengths of the current study are its randomized controlled design, the use of a well- defined perioperative management protocol, the relative large sample size of patients with a rare disease, and the comprehensive prospective data collection. Our study also has some limitations. Preoperative blood pressure targets were achieved in only a minority of the participants. In particular, a large majority did not reach the strict upright blood pressure target. It should be noted, however, that these blood pressure targets are mainly based on expert opinion and have never been evaluated prospectively before. Of potential interest, we showed that a preoperative supine blood pressure <130/80 mmHg is associated with less hemodynamic instability while an upright systolic blood pressure <90 mmHg is associated with more hemodynamic instability, as has been suggested

previously (10). This finding could guide future recommendations concerning preoperative blood pressure targets. Furthermore, we did not include a placebo group, and study drugs were provided in an open-label fashion. Incorporation of a placebo arm was, however, considered to be unethical in view of current guidelines recommending pretreatment with an α-adrenergic receptor blocker (5, 34). Moreover, retrospective studies questioning the importance of pretreatment are confounded by the administration of antihypertensive agents other than α-adrenergic receptor blockers and lack crucial information on perioperative administration of vasoactive drugs (35, 36). We chose for an open label design because blinded administration of the study drugs would have required a double-dummy design with the ensuing risk of insufficient medication adherence due to the relatively large number of placebo and verum drugs that would need to be ingested by the participants.

In conclusion, the duration of blood pressure being outside the target range during surgical resection of a PPGL was not different after preoperative treatment with either phenoxybenzamine or doxazosin. Phenoxybenzamine prevented high blood pressure more effectively and provided more hemodynamic stability during surgery. These data support the preoperative use of phenoxybenzamine in patients with a PPGL.

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Supplementary appendix

Supplementary methods

The difference in primary endpoint or in the HI-score was compared between patients with or without an intra-operative or postoperative cardiovascular complication. The following predictors of both the primary endpoint and the HI-score were considered: type of α-adrenergic receptor blocker, amount of additional antihypertensive medication expressed as total defined daily dosages,(1) American Society of Anesthesiologists (ASA) physical status, maximum tumor size on CT-scan or MRI, total plasma free metanephrines (i.e., the sum of plasma metanephrine and normetanephrine), total plasma catecholamines (i.e., the sum of plasma epinephrine and norepinephrine), surgical approach (laparoscopy or laparotomy), and supine mean arterial blood pressure on the day before surgery. Univariate correlations with a P-value <0.10 were included in a multivariable regression model using either the primary endpoint or the HI-score as dependent variable depending on which one demonstrated the largest effect size on cardiovascular complications. Achievement of preoperative blood pressure targets was applied sequentially as a co-variate with the following classification: 1) both supine and upright blood pressure within targets (i.e. supine BP <130/80 mmHg and upright SBP 110-90 mmHg), 2) supine blood pressure on target irrespective of upright blood pressure (i.e. supine BP <130/80 mmHg only), and 3) upright blood pressure below target irrespective of supine blood pressure (i.e. upright SBP <90 mmHg).(2, 3)

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Supplementary Table 1: Preferential intravenously administered drugs for intraoperative correction of hemodynamic variables.

Line of therapy	SBP>160 mmHg	MAP<60 mmHg	HR>100 bpm
1	magnesiumsulphate - 50 mg/kg loading dose - 2 g/h	volume therapy	esmolol - 10-20 mg/dosage - 50-300 µg/kg/min
2	phentolamine - 1-2 mg/dosage urapidil - 25-50 mg/dosage - 2-50 µg/kg/min	phenylephrine - 100 µg loading dose	In case of arrhythmias: magnesiumsulphate - 50 mg/kg loading dose - 2 g/h lidocaine - 1 mg/kg
3	nicardipine - 1-3 µg/kg/min	norepinephrine - 0.05-0.1 µg/kg/min	
4	sodium nitroprusside - 0.5-5 µg/kg/min nitroglycerine - 0.5-10 µg/kg/min	terlipressin - 1-3 mg ephedrine - 5 mg	

Supplementary Table 2: Thresholds and attributed points for the hemodynamic instability (HI) score.

Domain	HI-score component	Value	Score
Hemodynamic variables	Maximum SBP (mmHg)	<160	0
		160 - 179	1
		180 - 199	3
		≥200	7
	Time SBP >160 mmHg (%)	0	0
		0.1 - 1.0	1
		1.1 - 6.6	3
		≥6.7	7
	Minimum MAP (mmHg)	≥60	0
		50 - 59	1
		40 - 49	3
		<40	7
	Time MAP <60 mmHg (%)	0	0
		0.1 - 1.1	1
		1.2 - 4.1	3
		≥4.2	7
	Maximum HR (bpm)	<100	0
		100 - 119	1
		≥120	3
	Time HR >100 bpm (%)	0	0
		0.1 - 1.0	1
		≥1.1	3
	Minimum HR (bpm)	≥50	0
		40 - 49	1
		<40	3
	Time HR <50 bpm (%)	0	0
		0.1 - 1.7	1
		≥1.8	3
Volume therapy	Volume therapy (ml/kg/h)	0 - 84	0
		≤ 6.3	2
		6.4 - 9.7	6
		9.8 - 14.3	14
		≥14.4	30
Vasoactive drugs	Norepinephrine (µg/kg/h)	0	0
		>0 - 1.91	3
		1.92 - 4.20	9
		≥4.21	21
	Phenylephrine (µg/kg/h)	0	0
		>0 - 1.22	2
		1.23 - 3.72	6
		≥3.73	14

Supplementary Table 2: Continued

Domain	HI-score component	Value	Score
	Vasopressor other	No	0
		Yes	5
	Magnesiumsulphate (g/kg/h)	0	0
		>0-0.0131	3
		0.0132-0.0254	9
		≥0.0255	21
	Phentolamine (mg/kg/h)	0	0
		>0-0.0193	2
		0.0194-0.0356	6
		≥0.0357	14
	Vasodilator other	No	0
		Yes	5
	Esmolol (mg/kg/h)	0	0
		>0-0.1224	1
		0.1225-0.4463	3
		≥0.4664	7
	β-adrenergic receptor blocker	No	0
		Yes	3
Total			**0-160**

SBP: systolic blood pressure, MAP: mean arterial pressure, HR: heart rate.

Supplementary Table 3: Preoperative reported adverse events

	Phenoxybenzamine (*n*=68)	**Doxazosin (*n*=72)**	***P*-value**
Side effect - no. (%)			
Dizziness	28 (58.8)	30 (41.7)	0.06
Dry mouth	11 (16.2)	5 (6.9)	0.11
Dry eyes	2 (2.9)	1 (1.4)	0.61
Nasal congestion	24 (35.3)	8 (11.1)	<0.001
Fatigue	20 (29.4)	15 (20.8)	0.33
Headache	11 (16.2)	10 (13.9)	0.81
Palpitations	6 (8.8)	12 (16.7)	0.21
Abdominal distension	7 (10.3)	17 (23.6)	0.04
Obstipation	2 (2.9)	3 (4.2)	1.00
Dyspnea	2 (2.9)	5 (6.9)	0.44
Urinary incontinence	3 (4.4)	1 (1.4)	0.36
Peripheral edema	3 (4.9)	7 (9.7)	0.33
Miscellaneous	19 (27.9)	21 (29.2)	>0.99
Number of side effects - no. (%)			0.18
0	5 (7.4)	15 (20.8)	
1	19 (27.9)	18 (25.0)	
2	19 (27.9)	18 (25.0)	
3	10 (14.7)	10 (13.9)	
4	12 (17.6)	6 (8.3)	
5	3 (4.4)	3 (4.0)	
6	0 (0)	2 (2.8	

Data are reported as number of patients (%)

Supplementary Table 4: Univariate correlation between possible predictors of the primary end point and hemodynamic instability (HI) score.

	Primary endpoint		**HI-score**	
Predictor	**R_s**	**P-value**	**R_s**	***P*-value**
α-blocker (PXB vs DOX)	0.028	0.75	0.201	0.02
Amount of antihypertensive drugs	0.085	0.33	0.031	0.72
ASA physical status	0.061	0.50	-0.020	0.82
Tumor size	0.229	<0.001	0.289	<0.001
Total plasma metanephrines	0.295	<0.001	0.301	<0.001
Total plasma catecholamines	0.290	<0.001	0.234	<0.001
Surgical approach (LS vs LT)	0.068	0.43	0.092	0.29
Preoperative MAP	0.068	0.47	0.101	0.25

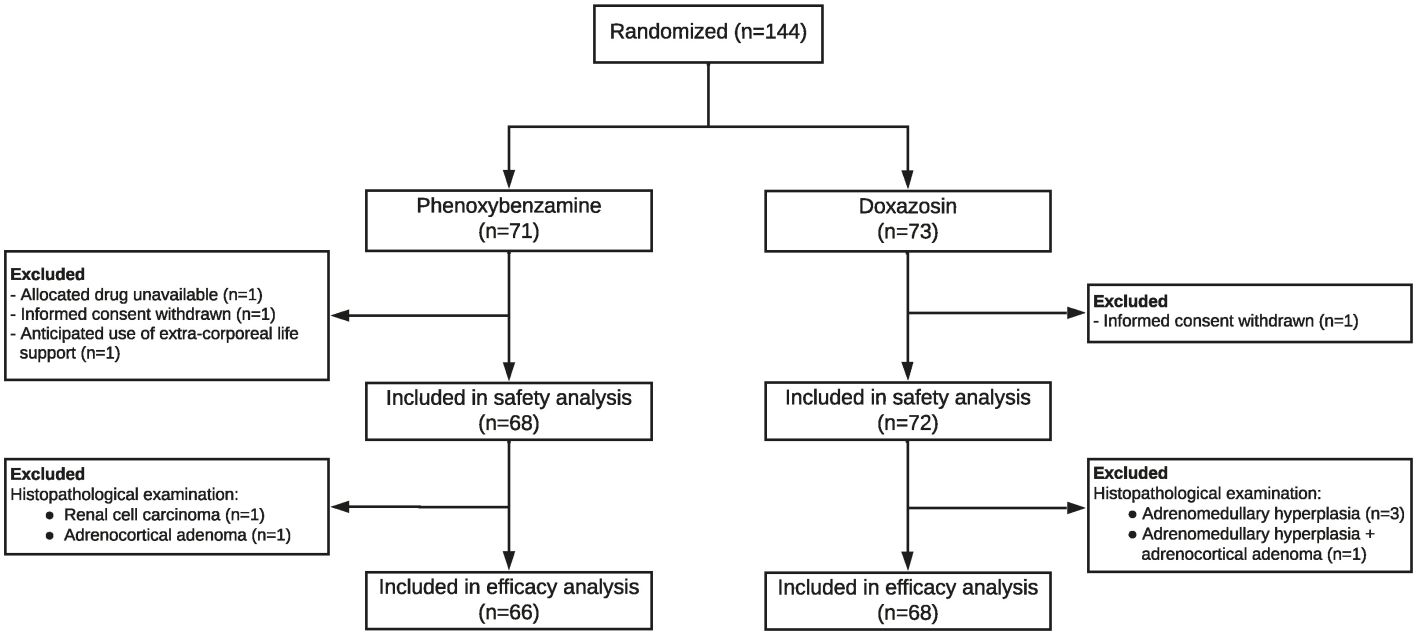
PXB: phenoxybenzamine, DOX: doxazosin, ASA: American Society of Anesthesiologists, MAP: mean arterial pressure, LS: laparoscopy, LT: laparotomy.

Supplementary Table 5: Multivariable regression models with the hemodynamic instability score as a dependent variable.

	Baseline model		Model 1		Model 2		Model 3	
Independent variable	**β**	**P-value**	**β**	**P-value**	**β**	**P-value**	**β**	**P value**
Randomization (PXB/DOX)	0.18	0.03	0.16	0.06	0.20	0.02	0.16	0.06
Tumor size (mm)	0.18	0.04	0.19	0.04	0.18	0.05	0.20	0.03
Catecholamines (nmol/L)	0.20	0.03	0.17	0.06	0.16	0.07	0.18	0.05
All BP targets achieved (no/yes)			-0.11	0.20				
Supine BP <130/80 mmHg (no/yes)					-0.18	0.04		
Upright SBP < 90 mmHg (no/yes)							0.19	0.02

PXB: phenoxybenzamine, DOX: doxazosin, mm: millimeter, BP: blood pressure, SBP: systolic blood pressure, β: standardized regression coefficient.
Baseline model: type of α-adrenergic receptor blocker, tumor size, and total plasma catecholamines (R 0.365, R^2 0.133).
Model 1: baseline model + achievement of supine BP <130/80 mmHg and upright SBP 110-90 mmHg on the day before surgery (R 0.371, R^2 0.137).
Model 2: baseline model + supine BP <130/80 mmHg irrespective of supine blood pressure on the day before surgery (R 0.394, R^2 0.156).
Model 3: baseline model + upright SBP <90 mmHg on the day before surgery (R 0.402, R^2 0.162).

Supplemental Figure 1: Flowchart of included patients.



Chapter 10

Summary and General Discussion

Summary

Chapter 1 provides a general introduction and defines the aims of the present thesis. The function of the adrenal glands is described with a focus on the biosynthesis of steroid hormones in the adrenal cortex and evaluation of steroidogenesis with urinary steroid profiling. In addition, the clinical evaluation of adrenal tumors is described. The last section focusses on the preoperative treatment of patients with pheochromocytomas or sympathetic paragangliomas (PPGL). The overall aim of this thesis is to improve diagnostic strategies that intend to differentiate clinically relevant adrenal tumors from those without clinical consequence and to optimize preoperative treatment in order to prevent hemodynamic instability during surgical resection of a PPGL.

Part I of this thesis focused on adrenal steroidogenesis and its relationship with lipoproteins. In **Chapter 2** we described the validation of urinary steroid profiling using a newly developed gas chromatography with tandem mass spectrometry detection method (GC/MS-MS). This new method demonstrated excellent results for all validation parameters and the specificity was improved compared to the previously applied GC-MS method. A main advantage of this new method is a reduction of the analysis time to less than 24 hours, making it suitable for high-throughput analysis. Reference values for all 33 analyzed steroid metabolites were determined in a population of 240 healthy subjects recruited from the Lifelines cohort stratified by age and sex. In addition, 40 women using oral contraceptive pills (OCP) were analyzed separately. The results demonstrated that the urinary excretion of almost all steroid metabolites is affected by age and sex, underscoring the need for age and gender specific reference values. Moreover, the use of OCP resulted in a lower excretion of progesterone and androgen metabolites and this factor should be taken into account in daily clinical practice.

In **Chapter 3** we determined the relationship between total glucocorticoid production (TGP) and plasma HDL-C levels, in view of previous findings suggesting that adrenal function may be attenuated in men with genetically determined low levels of plasma high density lipoprotein cholesterol (HDL-C).1 TGP was estimated as the sum of glucocorticoid metabolites that were determined in a 24-hour urine collection using GC/MS-MS. We analyzed these variables in 240 healthy subjects recruited form the Life Lines cohort who were stratified by age and sex. TGP tended to be increased in subjects with a low plasma HDL-C concentration according to NCEP-ATPIII criteria.2 Univariate analysis demonstrated that TGP was inversely

correlated with HDL-C. Multivariable linear regression analysis demonstrated that TGP was still inversely related to HDL-C after adjustment for a variety of metabolic syndrome components. These data do not support the hypothesis that adrenal function is attenuated in subjects with low plasma concentrations of HDL-C as previously suggested.

In **Chapter 4** we measured lipoprotein particle concentrations in 23 patients with primary aldosteronism. Blood samples collected during an adrenal vein sampling procedure from the inferior vena cava (IVC) and both adrenal veins were analyzed. We found that plasma HDL- C and particle concentrations of HDL and of LDL were not lower in the adrenal veins compared to the IVC, whereas ApoB tended to be lower in the adrenal vein samples. In a subset of 13 patients with an aldosterone producing adenoma in whom we expected to find the highest uptake, it was found that plasma ApoB concentrations were lower with a trend towards lower LDL particle concentrations in the adrenal vein. These observations suggest that cholesterol derived from circulating LDL particles may contribute to adrenal steroidogenesis, and that adrenal venous sampling is a feasible model to determine uptake of lipoproteins in the adrenal glands.

In **Part II** of this thesis we focused on diagnostic strategies for PPGL. In **Chapter 5** we performed a nationwide pathology study to identify all histopathologically confirmed cases of PPGL diagnosed between 1995 and 2015. A total of 1493 patients with PPGL were identified. We found that the age-standardized incidence rates for PPGL increased during the study period. Concomitantly, pheochromocytoma size decreased and age at diagnosis increased. Additionally, we conducted a systematic review of the literature in which we identified only three papers reporting on nationwide incidence rates of PPGL between 1949 and 1981 in North-West European countries. A comparison of these incidence rates showed that the observed increase in incidence started already several decades ago. These results might in part reflect the changing clinical practice with augmented use and improved accuracy of imaging and biochemical tests for detecting PPGL.

Chapter 6 concerned a retrospective multi-center study in which unenhanced CT-scans of 214 patients harboring 222 histopathologically confirmed pheochromocytomas were reevaluated by two independent radiologists. We found that only one pheochromocytoma demonstrated an unenhanced attenuation value ≤10 Hounsfield Units (HU). Moreover, the interobserver consistency and concordance were excellent. These data support the hypothesis

that biochemical testing to rule out a pheochromocytoma in patients with an adrenal incidentaloma is only indicated when the unenhanced attenuation value is >10 HU, and suggest that attenuation measurement for this purpose is well suited for implementation in general clinical practice.

In **Chapter 7** we confirmed the results of chapter 6 in a systematic review and meta-analysis, including 1167 pheochromocytoma cases from 31 studies. We found that the proportion of pheochromocytomas with an unenhanced attenuation value >10 HU was 0.990 (95% CI: 0.984-0.995). The negative predictive value was estimated to be close to 100%. A new diagnostic algorithm in which biochemical testing to exclude a pheochromocytoma is obviated in patients with an adrenal tumor and unenhanced attenuation value ≤10 HU was proposed. A probabilistic sensitivity analysis that modeled the costs of this diagnostic algorithm demonstrated a modest cost reduction compared to current diagnostic practice.

Part III of this thesis was focused on optimizing perioperative hemodynamic stability in patients with PPGL. In **Chapter 8** we described the development and internal validation of the hemodynamic instability score (HI-score). The premise of the HI-score is that both hemodynamic variables as well as interventions aiming to normalize hemodynamic variables (i.e. cardiovascular medication and fluid therapy) are important elements of overall hemodynamic instability. In a development cohort, consisting of patients that were expected to represent the entire hemodynamic instability spectrum, normalized threshold values of all hemodynamic and intervention variables were determined to assign scores. Subsequently, these scores were applied to the validation cohort in which we demonstrated that the HI- score was significantly different between surgical procedures associated with a low or high degree of hemodynamic instability. The HI-score provides a clinical tool which shows promise for future applications in both patient management and clinical research following further external validation.

Chapter 9 involved the first randomized controlled trial that compares the efficacy of two α- receptor antagonists in the preoperative treatment of patients undergoing resection of a PPGL. Patients were randomized between pretreatment with either doxazosin or phenoxybenzamine. The primary endpoint, defined as the cumulative percentage of intraoperative time with a systolic blood pressure >160 mmHg or a mean arterial pressure <60 mmHg, was not different between treatment groups. The overall degree of hemodynamic instability, assessed by

the HI-score, was higher in the doxazosin group. No differences in mortality or cardiovascular complications rates were observed between both treatment arms. Of note, patients with a cardiovascular complication had a significantly higher degree of hemodynamic instability. Based on secondary endpoints, the results of this trial suggest a greater efficacy of phenoxybenzamine in preventing intraoperative hemodynamic instability.

General discussion

Cholesterol trafficking into and within the steroidogenic cell is a complex process to study, since there are multiple pathways by which cholesterol can be delivered to the mitochondria for further processing (3). Cholesterol in adrenal cortical cells can be derived from i) *de novo* synthesis in the endoplasmic reticulum, ii) storage in intracellular lipid droplets, or iii) circulating lipoproteins. Studies from the 1960s and 1970s using radiolabeled cholesterol demonstrated that circulating cholesterol is the most important contributor to adrenal steroidogenesis (4). Both the LDL-receptor, that internalizes the entire LDL-particle, and the SRB1-receptor, that enables cholesterol import from the HDL-particle into the cell, are present on human steroidogenic cells (3-6). It is therefore conceivable that both HDL and LDL may play a role in cholesterol delivery needed for adrenal steroid biosynthesis. *In vivo* studies that investigated the relative contributions of both these classes of lipoproteins predominantly have been performed in rodents (4). However in rodents, HDL serves as the predominant lipoprotein class with lower levels of LDL compared to humans, in whom LDL is the predominant class with lower levels of HDL. Findings from these animal experiments, therefore, do not necessarily hold true for humans. In an attempt to directly determine the uptake of lipoprotein particles we used adrenal venous sampling in patients with primary aldosteronism. Of note, blood flow in the adrenal glands as well as concentrations of circulating lipoproteins are relatively high compared to steroid output, even in the context of ACTH stimulation (7). The rate at which cholesterol is extracted from circulating lipoproteins by the adrenal glands is, therefore, presumably low. Despite these challenges we were able to detect a consistent uptake of apoB and a trend towards uptake of LDL-particles. We regard this as a proof of concept and consider this approach feasible to asses lipoprotein uptake in humans. No evidence was found for HDL-C uptake or for a change in HDL particle size. The latter would have been expected given the mechanism of SRB1-mediated cholesterol uptake that directs the cholesterol cargo of HDL particles into the adrenal cortical cells leaving the HDL-particle itself intact. Of note in a subsequent study, non-diabetic patients derived from the same cohort of hyperaldosteronism patients, were found to have significantly lower levels of LDL particle and HDL particle concentrations compared to normotensive and hypertensive subjects (8). The mechanism that is responsible for this apparently unique lipoprotein profile in patients with primary aldosteronism is currently unclear.

The relationship between concentrations of circulating lipoproteins and steroid hormone production in humans is predominantly derived from patient populations

with certain genetic alterations in cholesterol metabolism and trafficking. For example, patients with abetalipoproteinemia or defects in the LDL receptor pathway were demonstrated to have normal basal cortisol production, but a mildly attenuated response to ACTH (9,10). This suggests that cholesterol supply from *de novo* synthesis or from circulating HDL is sufficient to ensure adequate cortisol production under basal conditions. In contrast, basal steroidogenesis was suggested to be attenuated in male patients with genetically low HDL-levels but responses to ACTH stimulation were normal (1). In women with genetically determined low HDL-levels the adrenal function was, however, normal (11). Additionally, both basal and stimulated adrenal function was decreased in a family with a mutation in the SRB1 receptor that facilitates extraction of cholesterol from HDL-particles into the steroidogenic cell. Together, these data suggest an important role for the HDL-SRB1 pathway in basal and stimulated steroidogenesis, but the evidence is somewhat equivocal. It should be noted that various methods were used to quantify steroid production. By application of a more precise technique we found a trend towards an increased glucocorticoid production in subjects with non-genetically determined low HDL levels. Thus, it seems likely that low HDL-C levels do not contribute to decreased glucocorticoid production under basal circumstances. Possibly, even low levels of HDL-C are sufficient to utilize the HDL-SRB1 pathway or the LDL pathway is utilized simultaneously. Further employment of the adrenal venous sampling model to compare subjects with the aforementioned genetic alterations under both basal and stimulated conditions may give further direction to this research question. Additionally, super- selective adrenal venous sampling might reduce venous dilution significantly (12). Alternatively, synthetic lipoproteins filled with, for example, radiolabeled cholesterol could be used to study adrenal lipoprotein utilization *in vivo* (13,14).

Overall our findings suggest that LDL might be important for steroidogenesis after ACTH stimulation, but we cannot exclude the possibility that HDL contributes as well. From an evolutionary point of view, it could be envisaged that multiple pathways are in place for such a vital physiological function as steroid biosynthesis and that, therefore, both LDL and HDL particles can be used to supply cholesterol to the adrenal glands.

Adrenal incidentalomas are by definition a direct consequence of the ever increasing utilization and improving performance of imaging techniques (15,16). This clear trend in the clinical application of various imaging modalities results in

the detection of more and smaller sized adrenal incidentalomas. Distinguishing between adrenal tumors that need specific treatment and benign non-functioning adenomas is, has therefore become an increasingly relevant clinical problem. When discussing the diagnostic approach of patients with an adrenal incidentaloma it is foremost important to consider how often an adrenal tumor has clinical consequences. In 2016, more than 1.7 million CT-scans were performed in the Netherlands (16). The adrenal glands are visualized in about two thirds of these. Assuming a 3- 10% prevalence of adrenal incidentalomas as reported in radiological studies, the number of patients with an adrenal incidentaloma is quite high (17,18). Reliable incidence rates are lacking, but it can be estimated that: 8500 patients with an adrenal incidentaloma should be annually identified in The Netherlands assuming that 1 in 4 CT-scans is performed in a unique patient and the adrenal incidentaloma frequency is 3%. A systematic review of the literature demonstrated that adrenocortical carcinomas, pheochromocytomas and metastases represent 8% (range: 1.2-11%), 7% (range: 1.5-14%) and 5% (range: 0-18%), respectively, of all adrenal incidentalomas.(19) Obviously, the frequency of aforementioned clinical relevant etiologies are not in agreement with their known incidence rates considering the prevalence of adrenal incidentalomas. For example, the annual incidence rate of adrenocortical carcinoma is just over 1 per million person-years (20). Assuming an adrenal incidentaloma frequency of 8500, 8% of which represent an adrenocortical carcinoma, then the incidence rate of adrenocortical carcinoma would be 40 per million person-years. The same line of reasoning applies to pheochromocytomas. We demonstrated that the incidence rate of pheochromocytomas is increasing over the years, but this disease should still be considered a rare disease. During the entire observation period, the incidence rate was the highest and increased the fasted in patients older than 50 years. This suggests an important contribution of incidental discovered pheochromocytomas, since this age group is most frequently scanned. However, the incidence rate we found is not even nearly in agreement with the 7% frequency of pheochromocytomas in adrenal incidentalomas. Of note, it is conceivable that not all pheochromocytomas are currently detected. Especially, oligosymptomatic pheochromocytomas are often discovered as adrenal incidentaloma and are therefore more likely to remain undetected despite a larger tumor size and higher metanephrines compared to hereditary and symptomatic cases (21).

Overestimation of the frequency of clinical significant adrenal incidentalomas is likely the result from several kinds of methodological shortcomings, in particular selection bias in the reported studies (22). About half of adrenal incidentalomas

that are visualized on CT are not mentioned in the radiology report (23). In addition, larger lesions are more often reported in specific terminology by the radiologist and these patients are more frequently referred for further analysis compared to non-specifically reported lesions (24). In real-life clinical practice, however, only 14% of patients with an adrenal incidentaloma that was reported in specific terminology underwent a complete biochemical and radiological workup (24). The smaller non- specifically reported lesions are less likely to be clinically relevant but are underrepresented in the majority of retrospective studies. However, size alone is a suboptimal predictor of hormonal hypersecretion (25). Therefore, the proportion of patients with a clinical relevant adrenal incidentaloma is overestimated but at the same time clinical relevant adrenal incidentalomas are currently missed. This may significantly influence the performance of various diagnostic tests that are applied in patients with an adrenal incidentaloma in daily clinical practice.

Many diagnostic algorithms for adrenal incidentalomas have been published over the years (19,26-33). A high diagnostic burden to evaluate the nature and hormonal function of adrenal incidentalomas is a common denominator of these guidelines. The level of evidence that supports these recommendations is in general either low or very low. The major factor of the low level of evidence is the retrospective design of the majority of studies that were taken into account to generate the guidelines. Nowadays, the overall trend in the diagnostic approach is towards less diagnostic tests and a shorter follow-up time (19). This makes sense from the perspective that at least 70%, and probably a much higher percentage, of all adrenal incidentalomas can be classified as benign non-functioning adenomas. This thesis contributes to the changing view in diagnostic approach by demonstrating that determination of metanephrines can be obviated in patients harboring an adrenal incidentaloma with an unenhanced attenuation value ≤10 Hounsfield Units (HU). Admittedly, this recommendation is also based mostly on retrospective data. However, selection bias appeared not to be a major confounder in our meta-analysis since we only looked to determine the sensitivity of the 10 HU cut-off value and the results, therefore, seem to be robust. Prospective validation would further strengthen this recommendation.

Another major factor in the diagnostic burden is the differentiation between malignant and benign disease. The current approach using an unenhanced CT-scan and if needed a wash-out CT-scan and FDG-PET scan is considered highly sensitive, but all modalities lack specificity (34-36). The low accuracy of malignancy risk assessment is underscored by studies demonstrating that more than one third of operated patients is eventually diagnosed with a nonfunctioning adenoma (30).

Therefore, there is a need for alternative, simple and more accurate diagnostic tests. Urinary steroid profiling seems a promising tool for this purpose.

Retrospective studies demonstrated its high accuracy to discriminate benign from malignant adrenocortical tumors (37,38). In this thesis we demonstrated the influence of age and sex for steroid metabolite excretion in a healthy population. Whether deviation from normal values points towards relevant disease could not be delineated from this study. Incorporation of the identified influencing factors into an integrated analysis of steroid hormone metabolites might improve the diagnostic accuracy and should be further investigated. Furthermore, prospective validation of urinary steroid profiling in an adrenal incidentaloma population is needed before widespread implementation in clinical practice. One such initiative is the Systematic Evaluation of adRENal tumors Discovered Incidentally – Prospectively Investigating the Testing Yield (SERENDIPITY) study that aims to include 1000 patients with an adrenal incidentaloma to determine the added value of a single urinary steroid profile compared to repeated CT-scans. Additionally, this study will be able to provide a comprehensive analysis of costs and quality of life associated with the adrenal incidentaloma work-up. Moreover, SERENDIPITY can provide a prospective validation of the accuracy of unenhanced CT-scanning to exclude a pheochromocytoma.

To conclude, the future challenge in the adrenal incidentaloma field is to develop and properly validate diagnostic tests that are accurate and simple to apply to a growing number of patients with a low pretest probability for clinically relevant disease.

Pretreatment of patients with an α-adrenergic receptor blocker before resection of a PPGL was first introduced around 1949 and has been part of clinical practice ever since (39). During surgical resection of a PPGL patients are at risk for a catecholamine crisis that can lead to hemodynamic instability, cardiovascular complications such as a cerebrovascular event or myocardial infarction, and ultimately death. The perioperative mortality rate has declined dramatically since the introduction of pretreatment from more than 25% to almost 0% nowadays (40-42). The cardiovascular complication rate is, however, still around 9% (42). Of note, it remains unknown whether the improvement in patient outcome is fully attributable to pretreatment because no placebo-controlled studies have been performed to date (43). Most likely, improvements of preoperative tumor

localization as well as anesthetic and surgical techniques also have contributed to the significant reduction in perioperative risk.

The Pheochromocytoma RandomisEd Study Comparing adRenoreceptor Inhibiting agents for Preoperative Treatment (PRESCRIPT) study was initiated to compare efficacy of different types of α-adrenergic receptor antagonists. No randomized controlled trial has ever been undertaken on this topic, most likely due to the many challenges associated with the rarity of this disease. The PRESCRIPT-trial focused on intraoperative hemodynamic variables as outcome measures. Both mortality and cardiovascular complications would have been superior endpoints, but are unrealistic considering the required number of patients in relation to the low incidence rate of PPGL. For example, more than 1000 patients would need to be studied to demonstrate a relative reduction of 50% in the number of cardiovascular complications. The relevance of the chosen study endpoints is underlined by previous extensive observations that demonstrated an association between hemodynamic variables and adverse postoperative outcomes in various patient populations that underwent general anesthesia (44-50). Moreover, almost every patient experiences blood pressure fluctuations during PPGL resection (51). We found that the cumulative duration outside the predefined intraoperative blood pressure targets, the primary endpoint of the trial, was not different between phenoxybenzamine and doxazosin. A favorable effect of phenoxybenzamine on the duration of hypertension was obscured in the composite primary endpoint due to the relative large contribution of hypotension. In agreement with this, analysis of the secondary endpoints demonstrated less severe hypertension and a lower requirement of vasodilating drugs in patients pretreated with phenoxybenzamine.

It should be noted that many variables can be assessed as a marker of hemodynamic instability, but individually they do not capture the full picture. Moreover, many of these variables interact with each other which complicates a straightforward analysis. For example, if a patient becomes hypotensive and is, subsequently, administered a vasopressive drug, then the blood pressure is likely to normalize. However, a normal blood pressure with the support of vasoactive drugs does not represent a physiologically normal hemodynamic state. Although a frequently used term (i.e. >13,000 PubMed citations) the definition of hemodynamic (in)stability is not used in a uniform matter (52). In an effort to assess the overall degree of hemodynamic instability we developed the hemodynamic instability score (HI- score) in which many relevant variables are integrated into a single clinical score. The methodology we used for the development is straightforward and the

population was representative. The validation of this score is, however, complicated by the lack of a gold standard for hemodynamic instability. The added value of this score should be determined in future studies evaluating the relationship with, for example, postoperative adverse events or mortality in independent cohorts of patients that underwent general anesthesia. The application of the HI-score can theoretically be extended to any situation in which hemodynamic instability occurs, such as intensive care patients or post-anesthesia patients. Separate validation for these applications can be recommended. Commonly used cardiovascular drugs may vary between different patient populations and this should be taken into account when applying the HI-score. If necessary the point distribution of cardiovascular drugs should be modified. Of note, we also applied the HI-score to the PRESCRIPT-trial, after incorporating vasodilating drugs. We found an increased HI-score in patients who suffered from a postoperative cardiovascular complication. This observation should be regarded as the first external validation of the HI-score. In addition, HI-scores were lower in patients pretreated with phenoxybenzamine in accordance with other secondary outcome measures.

Taken all together, the PRESCRIPT-trial should be considered an inconclusive study on the primary endpoint, but secondary endpoints clearly suggest an advantage for phenoxybenzamine. We propose that this drug should be preferably used in the pretreatment of patients with PPGL. Limited availability due the narrow indication of phenoxybenzamine and associated high (unreimbursed) costs may hamper its use in daily clinical practice. Some authors even question the necessity for pretreatment altogether (43,53). It should, however, be realized that the data that is presented against pretreatment is heavily biased. For example, it is unclear how it was decided whether a patient would receive pretreatment, a large proportion of ''non-pretreated'' patients did receive other antihypertensive agents than α- receptor antagonists, details of intraoperative blood pressure deviations were poorly reported and no meaningful data on the amount of vasoactive drugs were provided (53). Moreover, the absence of a difference in complication rate lacks sufficient power for a meaningful interpretation. Furthermore, a longer duration of systolic blood pressure <80 and >200 mmHg has been demonstrated in untreated patients (54). However, it might be possible that not all patients benefit from pretreatment. An individualized approach to pretreatment should be substantiated by a reliable risk assessment that identifies patients with a low or high risk of hemodynamic instability. In an effort to delineate contributing factors we were able to explain only 15% of the variance in intraoperative hemodynamic instability by means of an exploratory analysis of the PRESCRIPT data. This

underlines the unpredictable hemodynamic consequences of PPGL and challenges the implementation of individualized pretreatment. Other potential influencing factors should be studied further to improve our understanding of the mechanisms that drive hemodynamic instability in patients with PPGL. For example, differences in catecholamine metabolism and secretion, single nucleotide polymorphisms that affect α-receptor activity, and downregulation of α-receptors might influence hemodynamic instability. A biobank containing pheochromocytoma tissue and DNA-samples of PRESCRIPT participants provides several opportunities to address these fascinating research questions.

Conclusion

The data in this thesis help to improve our understanding of adrenal tumors with a focus on diagnostic strategies and patient management. Ongoing efforts to enhance the discrimination between clinically relevant adrenal tumors and those without clinical consequences are supported by the development of an alternative diagnostic strategy. In addition, the findings described in this thesis should further optimize perioperative management of patients with PPGL, and may help to pave the way for a more comprehensive assessment of hemodynamic instability.

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Chapter 11

Nederlandse samenvatting

Nederlandse samenvatting

Hoofdstuk 1 omvat een algemene introductie en de doelstelling van dit proefschrift. De biosynthese van steroïdhormonen in de bijnierschors wordt uitgelegd alsmede de evaluatie van dit proces door middel van urine steroïdprofilering. Daarnaast wordt de klinische evaluatie van bijniertumoren beschreven. Vervolgens wordt ingegaan op de preoperatieve behandeling van patiënten met een feochromocytoom of sympathisch paraganglioom (PPGL).

De doelstelling van dit proefschrift is het verbeteren van diagnostische strategieën die gebruikt worden om klinisch relevante bijniertumoren te onderscheiden van bijniertumoren die geen verdere behandeling behoeven en het optimaliseren van de preoperatieve behandeling die gericht is op het beheersen van intra-operatieve hemodynamische instabiliteit bij patiënten met een PPGL.

Deel I van dit proefschrift richt zich op steroïdsynthese in de bijnierschors en de relatie hiervan met lipoproteïnen. In **Hoofdstuk 2** wordt de validatie van een nieuwe bepalingsmethode voor urine steroïdprofilering beschreven, waarbij gebruik gemaakt wordt van gaschromatografie met dubbele massaspectrometrie. Deze nieuwe methode laat uitstekende resultaten zien voor alle validatie parameters en een hogere specificiteit in vergelijking met de voorheen gebruikte methode waarbij enkelvoudige massaspectrometrie werd toegepast. Een belangrijk voordeel van de nieuwe methode is een sterke afname in de doorlooptijd tot minder dan 24 uur. Hierdoor wordt grootschalige toepassing van deze techniek mogelijk. Referentie waarden van alle 33 geanalyseerde metabolieten werden bepaald in een groep van 240 gezonde vrijwilligers uit het Lifelines cohort, gestratificeerd op basis van geslacht en leeftijd. Daarnaast werden referentiewaarden bepaald bij 40 vrouwen die orale anticonceptie (OAC) gebruikten. De resultaten lieten zien dat de excretie van vrijwel alle steroïdmetabolieten wordt beïnvloed door geslacht en leeftijd. Daarnaast bleek dat het gebruik van OAC resulteerde in een significant lagere excretie van progesteron en androgeen metabolieten. Dit betekent dat er met al deze factoren rekening moet worden gehouden bij de interpretatie van het urine steroïdprofiel.

In **Hoofdstuk 3** hebben we het verband onderzocht tussen totale glucocorticoïd productie (TGP) en plasma hoge dichtheids lipoproteïne (HDL) cholesterol concentraties. Eerder onderzoek liet namelijk zien dat de bijnierfunctie verminderd is bij mannen met een genetisch bepaalde lage plasma HDL concentratie. TGP werd gedefinieerd als de som van glucocorticoïd metabolieten gemeten in een

urine steroïdprofiel. In totaal werden 240 gezonde vrijwilligers uit het LifeLines cohort onderzocht die waren gestratificeerd op basis van leeftijd en geslacht. De resultaten lieten zien dat TGP enigszins verhoogd was in personen met een lage plasma HDL cholesterol concentratie. TGP bleek negatief gecorreleerd te zijn met plasma HDL cholesterol concentraties na correctie voor verscheidene componenten van het metabool syndroom. Deze resultaten zijn in tegenspraak met eerdere bevindingen dat de bijnierfunctie verminderd is bij personen met een lage plasma HDL-cholesterol concentratie.

In **Hoofdstuk 4** hebben we lipoproteïne deeltjes concentraties bepaald bij 23 patiënten met primair hyperaldosteronisme met als doel de opname van deze lipoproteïnen in de bijnier te bepalen. Bloedmonsters uit beide bijniervenen en vena cava inferior (VCI) werden verkregen door middel van een bijniervene sampling procedure. Plasma HDL cholesterol en deeltjes concentraties van HDL en lage dichtheids lipoproteïnen (LDL) waren niet lager in de bijniervenen in vergelijking met de VCI. Apolipoproteïne-B concentraties lieten een trend zien richting een lagere concentratie in de bijniervenen. In de groep waarin we hadden verwacht het grootste concentratie verschil te vinden, namelijk een subgroep van 13 patiënten met een unilateraal aldosteron producerend adenoom, bleek dat in de bijniervene aan de zijde van het adenoom de apolipoproteïne-B was verlaagd met een trend voor een lagere LDL deeltjes concentratie. Deze resultaten suggereren dat cholesterol afkomstig uit circulerende LDL deeltjes een rol zou kunnen spelen bij steroïdsynthese in de bijnierschors en dat de gebruikte methode toepasbaar is om opname van lipoproteïnen in de bijnier te kunnen meten.

Deel II van dit proefschrift richt zich op strategieën voor het diagnosticeren van PPGL. In **Hoofdstuk 5** beschrijven we ons onderzoek naar trends in de incidentie van PPGL. Alle histopathologisch bevestigde casus in Nederland tussen 1995 en 2015 werden gezocht. In totaal werden 1493 patiënten met PPGL geïdentificeerd. De leeftijd gestandaardiseerde incidentie van PPGL steeg gedurende de studieperiode. Tegelijkertijd nam de tumorgrootte af en steeg de leeftijd op het moment van de diagnose. In een aanvullende systematische literatuurstudie vonden we slechts drie studies die nationale incidentie cijfers van PPGL rapporteerden tussen 1949 en 1981. Een vergelijking van de uitkomsten van deze studies met de huidige resultaten liet een stijging zien van de PPGL incidentie sinds enkele decades. Mogelijk wordt dit verklaard door een toename in het gebruik en een verbeterde kwaliteit van zowel beeldvormende technieken als biochemische testen voor het diagnosticeren van PPGL.

Hoofdstuk 6 omvat een retrospectieve multicenter studie waarbij de precontrast CT-dichtheid van in totaal 222 feochromocytomen onafhankelijk werd bepaald door twee ervaren radiologen. We vonden slechts één feochromocytoom met een dichtheid ≤10 Hounsfield Units (HU). Daarnaast bleek dat de consistentie en concordantie in de dichtheidsmetingen tussen de radiologen uitstekend was. De resultaten ondersteunen onze hypothese dat biochemische testen om een feochromocytoom uit te sluiten bij patiënten met een bijnierincidentaloom alleen noodzakelijk zijn wanneer de CT-dichtheid >10 HU is. Daarnaast lijkt deze methode geschikt voor implementatie in de dagelijkse praktijk.

In **Hoofdstuk 7** bevestigen we de resultaten van hoofdstuk 6 in een systematische literatuurstudie en meta-analyse van 31 studies met 1167 feochromocytomen. We vonden dat de gepoolde proportie van pheochromocytomen met een CT-dichtheid >10 HU 0,990 (95% BI: 0,984-0,995) was. De negatief voorspellende waarde werd geschat op bijna 100%. We stelden een nieuwe diagnostische strategie voor waarin geen biochemische diagnostiek wordt gedaan om een feochromocytoom uit te sluiten bij patiënten met een bijnierincidentaloom en een CT-dichtheid ≤10 HU. Een probabilistische sensitiviteitanalyse liet zien dat de kosten van deze strategie lager waren in vergelijking met de huidige klinische praktijk.

Deel III van dit proefschrift richt zich op het optimaliseren van hemodynamische stabiliteit tijdens operatieve verwijdering van een PPGL. In **Hoofdstuk 8** beschrijven we de ontwikkeling en interne validatie van de hemodynamische instabiliteit score (HI-score). De presumptie bij de ontwikkeling van deze HI-score was dat zowel hemodynamische variabelen als interventies, die tot doel hebben om deze hemodynamische variabelen te corrigeren (bijv. cardiovasculaire medicatie of vochttoediening) belangrijke determinanten zijn van totale hemodynamische instabiliteit. In een ontwikkelingscohort bestaande uit patiënten waarvan we verwachtten dat ze het gehele hemodynamische instabiliteitsspectrum zouden vertonen, werden genormaliseerde afkapwaardes bepaald voor elke geïncludeerde variabele. Hieraan werd vervolgens een score verbonden. De hierbij ontwikkelde HI-score werd vervolgens getest in een validatiecohort met twee patiëntgroepen die een chirurgische ingreep ondergingen die geassocieerd was met ofwel een hoge ofwel een lage mate van hemodynamische instabiliteit. De HI-score bleek significant verschillend tussen deze twee groepen hetgeen illustreert dat variatie in hemodynamische instabiliteit betrouwbaar kan worden bepaald.

Hoofdstuk 9 omvat het eerste gerandomiseerde en gecontroleerde onderzoek waarin de werkzaamheid van twee verschillende α-receptor antagonisten voor de preoperatieve voorbereiding van patiënten met PPGL is onderzocht. Patiënten werden behandeld met fenoxybenzamine of doxazosine. Het primaire eindpunt, gedefinieerd als de cumulatieve duur van systolische bloeddruk >160 mmHg en gemiddelde arteriële bloeddruk <60 mmHg, was niet verschillend tussen de twee groepen. De mate van hemodynamisch instabiliteit, die werd gekwantificeerd met behulp van de HI-score, was hoger in de doxazosine groep. Er was geen perioperatieve mortaliteit en de frequentie van cardiovasculaire complicaties was niet verschillend tussen beide groepen. Patiënten die een cardiovasculaire complicatie ontwikkelden hadden een hogere mate van hemodynamische instabiliteit. Op basis van secundaire eindpunten laat deze studie zien dat fenoxybenzamine effectiever is in het voorkomen van intra-operatieve hemodynamische instabiliteit.

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Dankwoord

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List of publications

List of publications

Buitenwerf E, Berends AM, van Asselt AD, Korteweg T, Greuter MJ, Veeger NJ, Links TP, Dullaart RP, Kerstens MN. Diagnostic accuracy of CT imaging to exclude pheochromocytoma: a systematic review, meta-analysis and cost analysis. Mayo Clin Proc 2019 Accepted.

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